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The possibility that a combination of exposures to organophosphate esters (OPs) and the carbamate pyridostigmine bromide (PB) would lead to nerve damage in Gulf War veterans was studied by treating hens and mice with known neuropathic OPs diisopropyl fluorophosphate (DFP), triorthocresyl phosphate (TOCP), sarin and with PB. Subthreshold and threshold levels inducing nerve damage were established in hens repeatedly treated with TOCP and DFP. Multiple doses of sarin did not result in organophosphate induced delayed neuropathy (OPIDN). PB breached the blood/brain barrier at high lethal doses, but apparently not at low dose levels. TOCP did not induce OPIDN in mice at levels 85 times higher than levels that induced OPIDN in hens. Evidence was not obtained that PB enhanced TOCP induced neural damage in the hen. However, evidence was obtained that PB and DFP together caused neuropathological changes not seen after equivalent treatments with DFP alone. The study revealed additive effects of the OPs and PB on cholinesterase activity in brain that were not evident on the morphological or overt symptom levels. In summary, the data do not support the hypothesis that low level exposures to neuropathic OPs and to PB result in OPIDN.

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INTRODUCTION

It has been proposed that organophosphates (OPs) and carbamates may have acted together in triggering low-grade neurological syndromes in a small cohort of Gulf War veterans (Jamal *et al.*, 1996; Haley and Kurt, 1997). There have been suggestions, for example, that prophylactic use of pyridostigmine bromide (PB) may have increased susceptibility to OP compounds (pesticides, nerve agents) present in the S.W. Asia theater of operations (Abou-Donia *et al.*, 1996). Some allied troops may have been exposed to low airborne concentrations of sarin in Coalition-occupied Iraq, and there is concern that such agents may be used offensively in future military and civilian conflicts. Recent experience with civilian exposures to sarin in Japan (Yokoyama *et al.*, 1998) have challenged the widely held belief that sarin is unable to trigger the peripheral neuropathy that follows OP-induced actions on neuropathy target esterase (NTE). High doses of sarin (30-60 times the LD_{50}) are known to bring about organophosphate induced delayed neuropathy (OPIDN) in the chicken (Davies *et al.*, 1960; Gordon *et al.*, 1983). Additionally, the low-grade neurological syndromes reported in Gulf War veterans have been interpreted as mild forms of OPIDN (Haley and Kurt, 1997). OPIDN is a central and peripheral nervous system (CNS-PNS) distal axonopathy that is readily induced in hens treated with diisopropyl fluorophosphate (DFP) or tri-ortho-cresylphosphate (TOCP). TOCP has not been reported to induce an experimental myopathy associated with sarin, PB and other inhibitors of acetylcholinesterase attributed to excess acetylcholine (AChE; Dettbarn, 1984).

A goal of this research is to examine the hypothesis that PB may promote sarin's abilities to induce muscle damage and axonal degeneration. The study is directed toward the following specific questions: (1) Will single and repeated low-level exposures to sarin and PB produce neurological and neuromuscular damage, respectively, in selected laboratory species chosen for their demonstrated sensitivity to OPIDN (hen) and ACh-induced myopathy (rodent)? (2) Even though motor endplates are vulnerable to damage after acute cholinesterase (ChE) inhibition and consequent excessive ACh receptor stimulation, will sensory nerve twigs and endings be more vulnerable than equivalent parts of motor fibers at the commencement of OPIDN? (3) Will PB exacerbate sub-clinical, sarin-induced CNS-PNS axonopathy when the carbamate is administered after the OP?

BODY

Materials

Strain Cd1 male mice were purchased from Charles River. White leghorn hens were purchased from Lakeview Farms as day-old chicks and raised in Animal Science Department facilities at UC Davis. Dilute sarin (XGB) was supplied by the US Army Medical Research Institute of Chemical Defense. Diisopropyl fluorophosphate (DFP), paraoxon (PO), atropine sulfate, and 2-pralidoxime (2-PAM) were purchased from Sigma Chemical Co. Tri-ortho-cresyl phosphate (TOCP) was purchased from Acros Organics.

Methods

Animal Injections

Chickens were dosed by intramuscular (im) injection in the pectoral muscle. Chickens that received TOCP were dosed by subcutaneous (sc) injection at the inner thigh. Birds dosed with high levels of OP compounds were given 20 or 50 mg/kg atropine sulfate immediately prior to the OP injection to prevent acute cholinergic effects. If necessary, 2-pralidoxime (2-PAM; 50 mg/kg) was administered intravenously (i.v.) to further alleviate acute effects (Wilson, *et al.*, 1988).

Mice were dosed by s.c. injection at the back of the neck. Atropine (1-2 mg/kg) and 2-PAM were administered as necessary to alleviate acute cholinergic effects of high OP doses such as to prevent convulsions and the consequent lack of oxygen to and damage in the brain that might confound the histopathology.

Sacrifice

Mice and chickens were sacrificed by cervical dislocation for biochemical assessment. Animals were anesthetized and perfused with buffered fixative through the ascending aorta (as described below) for morphological assessments.

Tissue Sampling for Biochemistry

Chicken blood was drawn into heparinized syringes from brachial (wing) or leg veins. The blood was centrifuged for 10 min at 1000 x g. The supernatant plasma was removed for ChE analysis (bird erythrocytes do not have cholinesterases). Brain and muscle were dissected after sacrifice. Tissues were kept on ice for immediate analysis, or frozen at -70°C for future study.

ChE Determinations

Samples were measured using the colorimetric method of Ellman *et al.* (1961), modified for use with an automatic microplate reader.

Neuropathy Target Esterase Determinations

Brain neuropathy target esterase (NTE) activity was determined by the colorimetric method of Johnson (1977) as modified by Wilson's laboratory (Mackay, *et al.*, 1996) from that of Correll and Ehrich (1991).

Protein Determinations

Protein was measured by the method of Lowry (1951).

OPIDN Clinical Assessment

Chickens were observed in their cages for standing and walking behavior. Observations were also made while prompting stationary birds to move by approaching or reaching towards them. Birds were characterized as being in one of five clinical states. 1: normal. 2: loss of coordination; stumbles; rests on hocks, but is able to stand. 3: leg weakness; bird rests and moves on its hocks; is unable to stand. 4: paralysis; legs are held out straight; bird can move by back-pedaling; locomotion limited. 5: moribund; bird falls to its side; cannot keep its body upright; cannot move about.

Morphological and Semi-quantitative Analyses

Animals were anesthetized, heparinized, the chest and heart opened, and perfused through the ascending aorta with 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4) for 10 sec followed by 5% glutaraldehyde in the same buffer for 10 min. Perfused animals were sent from the University of California at Davis (UC Davis) to Oregon Health & Science University (OHSU) for further processing. Selected tissues were dissected (Table 1). Tissues were placed in 0.1M sodium phosphate (pH 7.4), postfixed with 2% osmium tetroxide in the same buffer, dehydrated in ascending concentrations of ethanol, and embedded in Spurr's epoxy resin. One-micrometer-thick sections were stained with 1% toluidine blue and examined by bright-field microscopy. Thin sections were prepared as needed from sensory and motor nerve terminals, treated with 2% uranyl acetate and 1% lead citrate, and examined with a JEOL 100CX transmission electron microscope. Semi-quantitative light-microscopic assessment of neuropathological changes was made by two independent observers working without knowledge of the treatment schedule.

Observed changes were scored as follows: 0 = normal no observable pathology; 1 = normal appearance or non-specific changes (technically-induced); 2 = scattered abnormal cells (1-4 per low-magnification field); 3 = moderate numbers (5-8) of abnormal cells per low-magnification field, mostly early pathology observed; 4 = abnormal cells common and advanced pathology observed; 5 = advanced alterations, cellular destruction and cell loss. The assessment focused on the distal regions of peripheral nerves because reproducible changes can be detected there early in the disease process. The other tissue samples prepared in blocks are available if further assessment is required.

Animal dosing, perfusion, biochemistry and toxicology were carried out at the Institute of Toxicology and Environmental Health (ITEH) and the Department of Animal Science, located at UC Davis under the direction of P.I. Barry Wilson. Morphological assessment of sample tissues was carried out under subcontract at the Center for Research on Occupational and Environmental Toxicology (CROET), located at OHSU under the direction of co-P.I. Peter Spencer.

Task One: This consisted of "scoping" trials to establish appropriate dose/response ranges for sarin and control chemicals (DFP, TOCP and paraoxon).

Control Chemical Doses in Mice
Cd1 mice were dosed in groups of 3 with 1.5 mg/kg diisopropyl fluorophosphate (DFP) or 0.4 paraoxon, via s.c. injection (saline vehicle). One paraoxon-treated mouse died 25 minutes after dosing. The other mice were sacrificed 20 hours after dosing. Brain enzyme levels are summarized in Table 2.

High DFP Doses in Mice
Groups of 3 Cd1 mice were pretreated with atropine (40 mg/kg) 15 minutes prior to dosing with DFP, via s.c. injection (saline vehicle). Doses were at lethal levels: 1 at 3 mg/kg died after 2.4 hours; 2 at 4.5 mg/kg died after 1.6 hours; 2 at 6 mg/kg died after 0.5 hour. Mice showing acute cholinergic symptoms were given atropine (40 mg/kg) and 2-pralidoxime (175 mg/kg), but antidotes were unsuccessful. The remaining mice were sacrificed approximately 48 hours after dosing. Brain enzyme levels are summarized in Table 3.

In vitro Inhibition of Brain Enzymes
Homogenates of mouse and chicken brain were exposed to varying concentrations of sarin (XGB) and pyridostigmine bromide (PB).

AChE: For sarin inhibition, the approximate IC_{50} value)concentration (that which inhibited 50% of the activity was 4.0×10^{-8} M for mouse brain AChE and 2.7×10^{-8} M for chicken brain AChE (Figure 1). For PB inhibition, the approximate IC_{50} value was 9.1×10^{-8} M for mouse brain AChE and 7.6×10^{-8} M for chicken brain AChE (Figure 2).

NTE: For sarin inhibition, the approximate IC_{50} value was 6.4×10^{-7} M for chicken brain NTE (Figure 3). There was no inhibition of chicken brain NTE by pyridostigmine at concentrations up to 0.1 M (Figure 4).

Combination: Brain homogenates were treated with a combination of sarin (over a range of concentrations) and pyridostigmine (at a fixed concentration). The homogenates were incubated with either sarin or pyridostigmine for one hour at room temperature, and then the other chemical was added for an additional one hour incubation. The test was done in this way to look for effects such as reactivation of AChE, which could be dependent on the order the compounds were added.

Pyridostigmine at a concentration of 3×10^{-8} M inhibited mouse brain AChE by 70%. Sarin inhibited AChE in the same manner as when tested alone. The combination had no appreciable observed synergistic or antagonistic interactions; inhibition of AChE was additive (Figure 5).

Pyridostigmine at a concentration of 10^{-2} M had no effect on chicken brain NTE. Inhibition of NTE by sarin was the same in the presence or absence of pyridostigmine (Figure 6).

OPIDN Trial: DFP Treatment in Chickens

The purpose of this trial was to confirm DFP treatment levels that we have found to induce OPIDN in past studies. Four chickens were dosed i.m. with 300 μg/kg DFP (freshly purchased; saline vehicle). Each received 3 doses over a 6 day period (Wednesday, Friday, Monday; one bird received only 200 μg/kg of the second dose due to a miscalculation). Birds were observed for onset of OPIDN. First signs were observed on day 16, and 3 of the birds were at Stage 2 characterized by a lack of coordination and loss of balance while walking after 28 days. There was no progression after 43 days: 3 birds were at stage 2, and 1 bird was normal (stage 1; this was the bird which received the one reduced DFP dose). The OPIDN response was not as strong as expected. (In earlier studies, such doses resulted in birds that progressed to stages 4 and 5.) The plasma cholinesterase inhibition in the DFP-treated birds demonstrates that the dose was effectively delivered (Table 4). We interpret the results to signify in these studies that we are apparently at the dose that just causes OPIDN.

The above 4 birds as well as 2 untreated birds were perfused and processed for detailed morphological examination of susceptible regions of the nervous system. Examination of distal peroneal nerve, distal sural nerve and distal tibial nerve did not reveal a clear pattern of pathological changes. In sum, there was no strong evidence that the doses of DFP administered induced peripheral neuropathy.

OPIDN Trial: TOCP Treatment in Chickens

Given that the DFP trial described above did not demonstrate clear-cut neuropathological evidence of OPIDN, TOCP (another positive OPIDN control compound) was used to demonstrate that the chickens we were using were susceptible to OPIDN. Two birds were dosed s.c. with 500 mg/kg TOCP (neat) and observed for onset of OPIDN. The first signs were seen at day 14. On day 15, both birds were at stage 2; both at stage 3 on day 17; and stage 4 by day 21. The birds were perfused on day 23 and sent to OHSU for processing. There was evidence of a vacuolar primary axonal degeneration of large-diameter myelinated nerve fibers in the spinal cord and hindlimb peripheral nerves. The predominance of Wallerian-like degeneration in distal regions of peripheral nerves indicated the presence of a distal axonopathy. Small-diameter myelinated nerve fibers and unmyelinated nerve fibers were largely intact. These findings were consistent with the neuropathological picture (central-peripheral distal axonopathy) of the early phases of the delayed-onset peripheral neuropathy induced by single or repeated systemic treatment with TOCP.

PB Dosing Trial in Chickens

Several levels of PB were used to study the effects of PB on ChE and NTE enzyme activities. Pairs of birds were dosed i.m. with various levels of PB (water vehicle). Literature review suggested a level of 5 mg/kg would be tolerated. Chickens died of acute toxicity within 1 hr of treatment: two pairs died at 5 and 10 mg/kg PB, and 1 of 2 birds died at 2 mg/kg PB. The remaining 2 mg/kg PB-treated bird received 2-PAM. A pair given 1 mg/kg PB survived without administration of antidote. Plasma cholinesterase levels were severely depressed in the 2 mg/kg PB birds (Table 5). The surviving birds showed significant recovery of ChE 24 hours after

dosing. Figure 7 shows that there was no inhibition of brain NTE, but there was a dose-dependent inhibition of AChE in the brain. The lack of NTE inhibition agrees with our previous *in vitro* work with PB and NTE (Wilson *et al.*, 1999).

TOCP Dosing Trial in Chickens

Paired birds were treated s.c. with 0, 50, 100, or 150 mg/kg TOCP (neat) to identify a minimum dose that causes OPIDN to ascertain levels to use to assess effects of co-exposure to PB. Clinical signs were first seen on days 17-19 in the 100 mg/kg group. For the 150 mg/kg group, first signs were at days 21-26; and day 23 for the 50mg/kg group,. All birds progressed to stage 3 (and one bird to stage 4) in the 100 and 150 mg/kg groups. In the 50 mg/kg group, 1 bird showed stage 2 signs, while the other did not show any signs of OPIDN (stage 1, same as the untreated group). The birds were systemically perfused in groups of 4 on days 30 and 31 after treatment. Distal peroneal nerve, distal sural nerve and distal tibial nerve were sectioned, stained, mounted and examined (see Table 6 and Figure 8).

The morphology of the tissues was consistent with expectations of toxicity based on dose (i.e. a crude dose-response was evident). There was some variation among animals in the degree of response to low levels of TOCP (50mg/kg), and in an absence of a difference in degree of response to the two high levels of TOCP (100 and 150 mg/kg). This suggested that an intermediate dose (i.e. 75 mg/kg) would be adequate to detect modulation of response to TOCP induced by co-exposure to PB. Distal regions of the sural nerve, a sensory nerve, appeared to undergo degeneration earlier and to a more marked extent than equivalent regions of the tibial and peroneal nerves, both of which are mixed in function. Most toxic neuropathies that show the distal symmetrical pattern of retrograde axonal degeneration seen in OPIDN (and confirmed in the present study) are preceded by degenerative changes in sensory nerve fibers before they appear in equivalent regions of motor nerve fibers, and TOCP is apparently no exception. Note, however, that the clinical/behavioral changes seen in chickens are most likely attributable to changes in motor nerve fibers. Thus, sural nerve changes offer a potential sub-clinical biomarker of impending clinical OPIDN featured by limb weakness.

TOCP Dosing Trial in Chickens II

These trials sought to determine a threshold dose for TOCP-OPIDN for use in experiments seeking interactive (synergistic) effects of TOCP + PB in relation to OPIDN. Birds received a single s.c. dose (base of leg) of 0 mg/kg (n = 1), 50 mg/kg (n = 2), 75 mg/kg (n = 3), or 100 mg/kg (n = 2) undiluted TOCP. Animals were observed for clinical signs of OPIDN for up to 28 days. No clinical signs were observed in animals treated with 0, 50 (n = 2) or one with 75 mg/kg TOCP. The remaining 75 mg/kg and 100 mg/kg birds all showed stage 2 signs at 19-20 days, and stage 3 by 27-28 days. One of the 75 mg/kg birds progressed to stage 3 at day 21 and advanced to a stage intermediate between 3 and 4 at the end of the observation period.

Hens were perfused and sent to OHSU for histological assessment. Vulnerable regions of the peripheral and central nervous system were sectioned, mounted, stained, and scored (Table 7). Relative to controls, animals with clinical signs of OPIDN after treatment with 75 or 100 mg/kg TOCP showed pathological changes in peripheral nerves (distal regions > proximal regions), spinal cord and medulla oblongata (Figure 9). The pattern and distribution of neuropathology matched that expected for OPIDN.

TOCP Dosing in Mice: Trial I

Mice were dosed with the neuropathic positive control compound TOCP to investigate their susceptibility to OPIDN. Pairs of male Cd1 mice were given TOCP via s.c. injection at the base of the neck in doses of 0, 300, 500, 800 mg/kg in polyethylene glycol (PEG). Mice were

observed for 33 days during which there were no signs of limb weakness, a characteristic of OPIDN. Sores (redness and loss of hair) were observed 10 days after treatment at the injection site in all treatment groups. The sores were noticeably healing one week later. These mice were perfused at the end of the observation period and shipped to OHSU for histological examination. Unfortunately, the mice were packed with too many frozen cold packs and were frozen when received, making them unsuitable for histological examination.

TOCP Dosing in Mice: Trial II

The mouse trial was repeated using higher levels of TOCP since no clinical signs of OPIDN were observed in the first trial. Pairs of male Cd1 mice were given a single s.c. injection at the base of the neck of 0, 800, 1600, or 3200 mg/kg TOCP in corn oil vehicle. One animal treated with 3200 mg/kg was found dead one week after treatment, apparently killed by the other mouse treated at the same level. The mice were observed for 25 days after treatment, and no limb weakness was observed. Animals were perfused and shipped to OHSU in an inner container surrounded by foam packing material; they were received in good condition. Seven animals were processed for histological examination. Focus was placed on vulnerable distal regions of the tibial, peroneal and sural nerves. All samples of which were within normal limits in all animals (Table 8).

TOCP Mouse Trial III

Pairs of mice received a single s.c. injection at the base of the neck of 0, 3.2 g/kg (the highest dose in the previous trial), or 6.4 g/kg undiluted TOCP. The agent leaked from the injection site in one high-dose animal. A higher dose was planned but not used as it appeared a single dose with a larger volume of TOCP could not be administered to the mice.

Animals were observed for 49 days for signs of OPIDN. No clinical signs were seen. Six mice were perfused and sent to OHSU for histological assessment. Hindlimb nerves were sectioned, mounted, stained, and scored. Focus was placed on vulnerable distal regions of the tibial, peroneal and sural nerves, all of which were within normal limits in all animals (Figure 10). One animal (#11) treated with 6.4 g/kg TOCP showed minor abnormalities in the medial peroneal and distal tibial nerve (Table 9).

Taken together, the three TOCP Mouse Trials established that large single doses of TOCP fail to induce OPIDN in the strain of the species examined. In conclusion, therefore, mice proved unsuitable to execute study goals.

Task Two: To determine thresholds and relative dose-effect levels for biochemical and morphological end-points using multiple sarin exposures. This was intended to estimate the "highest no-effect dosage" (HNED) of sarin.

And

Task Three: Similar to Task Two, but the goal was to estimate the "highest no-effect dosage" (HNED) of PB.

In Vivo Enzyme Inhibition

Pairs of chickens were dosed three times i.m. over a period of 6 days with either 20 mg/kg atropine (ATR; in water, as a control), 250 µg/kg XGB, 200 µg/kg DFP, or 200 µg/kg paraoxon (PO; all OPs in saline) DFP- and PO-treated birds also received 20 mg/kg ATR i.m. prophylactically, while XGB-treated birds received ATR plus 50 mg/kg 2-PAM (both cholinergic antidotes). The XGB birds showed prominent acute symptoms (laying on side, with head down, unable to rise) after the first dose; symptoms subsided upon i.v. treatments with 78 mg of 2-PAM. For subsequent treatments, 2-PAM was administered immediately after the XGB doses. PO birds

showed acute symptoms, and 1 bird received an i.v. dose of 2-PAM after the second treatment. DFP- and ATR-treated birds showed minor (head shaking) or no symptoms.

Blood was drawn before, 1 hr and 24 hr after each treatment. Birds were sacrificed 24 hrs after the third treatment and the brains removed. Samples were stored frozen at -70°C. Blood was analyzed for cholinesterase (ChE), and brains were analyzed for both ChE and neuropathy target esterase (NTE).

The inhibition of brain enzymes showed that animals received toxicologically effective doses (Table 10, Figure 11). All three OPs caused high cholinesterase inhibition. The acute cholinergic symptoms correlated with the higher brain AChE inhibition of sarin and paraoxon. Sarin and DFP caused high NTE inhibition, exceeding the 80% inhibition threshold associated with OPIDN in acutely treated birds (Johnson, 1975). PO, which does not induce OPIDN, had no effect on NTE levels.

OPIDN Trial

Paired birds were dosed under the same paradigm outlined above (*In Vivo* Enzyme Inhibition). Both XGB birds died after the third dose. Birds showed pronounced symptoms, including mild convulsions, 20-30 minutes after dosing. They were given additional i.v. injections of 2-PAM, but died 5 and 20 minutes later (convulsions were observed in the latter bird). Another pair of birds was treated with a lower dose of XGB (200 µg/kg XGB), and an additional ATR control bird was treated. XGB-treated birds were strongly affected, so the dose was reduced further to 150 µg/kg XGB for the last 2 injections.

Blood ChE was measured prior to the first dose and at ~1 hr after each dose. Figure 12 shows that blood ChE was greatly reduced in OP-treated birds. The pattern was very close to that in the *in vivo* enzyme inhibition experiment. The blood ChE inhibition in the birds dosed with 150 and 200 µg/kg XGB was similar to that for birds dosed with 250 µg/kg XGB (Tables 10 & 11). Brain AChE activity of the dead birds was 95% inhibited compared to the level for ATR-treated birds (Table 10).

Birds were observed for 3 weeks after treatment, then perfused and sent to OHSU for histological assessment. There were no prominent clinical symptoms of OPIDN. One DFP treated bird showed occasional signs (falling back on hocks), but because of footpad swelling, these could not definitively be associated with OPIDN.

Nine chickens were perfused and sent to OHSU. Results are presented in Table 12. No clear pattern of pathological changes was found (Figures 13 & 14).

The lack of overt clinical or neuropathological signs of OPIDN was surprising. Both XGB- and DFP-treated birds showed marked NTE inhibition. The blood ChE inhibition showed that the birds dosed for the OPIDN trial received effective doses of the OPs, and the level of inhibition matched the earlier set of birds where the NTE inhibition was measured. This level of DFP has been used in our laboratory to induce OPIDN in the past.

Multiple Dosing Trial in Chickens

The purpose of this trial was to assess the effect of multiple doses and levels of XGB (sarin) or PB on ChE levels and clinical status. DFP was chosen as the positive OPIDN control because of prior experience with multiple low dosages of this agent inducing OPIDN (TOCP is an effective control only when single doses of agents are assessed).

Hens were dosed 20 times (5 days/week for 4 weeks) via injection into the pectoral muscle. Animals were treated in pairs with the following agents administered daily: atropine control (see below); 100 μg/kg DFP (positive OPIDN control); 25, 50 or 100 μg/kg XGB; 100, 250 or 500 μg/kg PB. The XGB-treated birds received 20 mg/kg atropine 15-30 minutes prior to sarin treatment. The atropine dose was raised to 50 mg/kg at the start of the second week. The atropine controls received the same dosage. The 100 μg/kg XGB birds also received 50 mg/kg 2-PAM i.v. (leg vein) immediately after the XGB dose; one of the 25 μg/kg XGB birds required 2-PAM on one occasion (at the start of the second week). The 2-PAM was given to birds that displayed marked acute toxic signs, including laying prone with the head down, often with eyes closed. One of the 100 μg/kg XGB birds was found dead the morning following the first dose (though it showed only mild signs such as sitting 2 hours after treatment). The other 100 μg/kg XGB bird died following the 5th dose. It had a mild convulsion while 2-PAM was being administered.

The body weight of the chickens over the course of the experiment is shown in Figure 15. PB-treated birds showed minor fluctuations in body weight. The atropine controls lost about 10% of body weight, recovering after dosing stopped. The XGB-treated birds had a rapid weight loss of 15-25%, recovering after dosing stopped. The DFP controls showed a steady loss over the first 37 days, even after dosing stopped. The bird showing signs of OPIDN lost close to 40% of its body weight by day 37 (the other DFP-treated bird was at 95% of its initial body weight).

As expected, OP and carbamate dosing caused inhibition of plasma ChE. Figure 16 shows the inhibition and recovery of ChE activity during the first week of dosing. Day 1 is the ChE activity 1 hour after dosing, showing inhibition of 75% and greater for most treatments. The lowest PB dose caused about 35% inhibition; atropine controls had no ChE inhibition. Day 2 showed recovery at 22 hours, before the next dose was given. Day 3 and 5 showed activity at 1 hour after dosing; levels were about the same as after the first dose. Day 8 showed recovery over the weekend. The recoveries were 60% and greater of initial activity; higher than the 1 day recovery values following the first dose. Figure 17 shows the plasma ChE activity post-dose over the course of dosing showing that the inhibition is fairly consistent over time. ChE inhibition did not progressively decrease with repeated dosing.

Only one of the DFP positive controls showed clinical signs of OPIDN. Stage 2 appeared on day 23 (during the last week of dosing; it received the last 4 doses after showing signs). OPIDN progressed to stage 3 on day 30, and to borderline stage 3/4 on 35. The bird remained at this stage until day 38, when it was perfused (along with one of the atropine controls) and shipped to OHSU for histological assessment. The remaining birds showed no clinical signs of OPIDN through day 50 (24 days after the last dose). These birds were perfused over days 50-52 and shipped to OHSU for histological assessment. Samples were taken from the fourteen animals. The most distal nerves were sectioned, mounted, stained, and scored (Table 13).

Sampled nerves of animals treated with sarin or atropine were within normal limits. One of the six animals treated with PB (Hen #9, 100 μg/kg) had an atypical distal peroneal nerve but the tibial and sural nerves were within normal limits. Of the two animals treated with DFP, one was within normal limits and the other (Hen #16, 100 μg/kg DFP) showed pathology in all three distal nerves. In sum, there was a variable individual animal response to 100 μg/kg DFP (x 20 doses). This result contrasted with the absence of OPIDN in animals treated with 200 μg/kg (x 3 doses; Task Two/Three: OPIDN Trial, above).

Task Four: Examine whether PB induces responses to sub-threshold doses of sarin or DFP when the carbamate is given before or after OP administration.

DFP and PB Combination Trial in Chickens

The first attempt to look at the effect of co-exposure of PB and an OP was to administer 0.5 mg/kg PB followed 4 hrs later by 0.5 mg/kg DFP to a pair of birds. One bird survived; the other died while the antidote 2-PAM was being administered. The additive ChE inhibition observed in the *in vitro* studies was considered when choosing the dose levels in this trial. Even so, the dose levels proved to be too high.

TOCP and PB Combination Trial in Chickens

The order of treatment of PB/OP exposures on OPIDN was studied. Lotti *et al.*, (1991) have shown phenylmethanesulfonyl fluoride (PMSF) protects against OPIDN when given before an OP, but promotes OPIDN if given after. For these studies, we decided to use TOCP as the neuropathic agent since a single dose can induce OPIDN. We reasoned that multiple DFP doses would confuse the issue of PB given before or after the OP.

Groups of 3 birds were given 0.5 mg/kg PB (i.m.); 200 mg/kg TOCP (s.c.); PB followed 4 hrs later by TOCP; or TOCP followed ~22 hrs later by PB. No antidote was necessary. Animals were observed for onset of OPIDN. First clinical signs were observed on day 15. Signs progressed to stage 3/4 in all TOCP-dosed birds by day 21 (except for one TOCP/no PB bird which only reached stage 2). PB had no observed effect on the clinical onset of OPIDN. Birds were perfused in batches of 4 on days 22, 23 and 25, and sent for morphological assessment of nervous tissue at OHSU. The most distal nerves were scored (Table 14).

The results showed again that the sural nerve was vulnerable to systemic TOCP treatments (see Figure 18 and Table 14). PB produced no morphological changes, as expected. Co-treatment with PB did not produce morphological evidence of modulation of TOCP-induced OPIDN. However, since the TOCP dosage was probably supramaximal (200 mg/kg) and the optimum dose for modulation studies appeared to be approximately 75 mg/kg (see TOCP Dosing Trial in Chickens under Task One), only evidence of down modulation of TOCP neurotoxicity would have been detectable.

DFP/PB Multiple Dosing Trial I

Groups of chickens (n = 3) were dosed with atropine (50 mg/kg; control group), 100 μg/kg PB, 100 μg/kg DFP, 200 μg/kg DFP, 100 μg/kg DFP + PB, or 200 μg/kg DFP + PB. All birds received the stated dose of atropine; PB treatment was given at the same time as the OP. Injections were given i.m. (pectoral); each agent was injected in a different area. Dosing was 5 days/week for up to 25 injections. Dosing ended when birds exhibited clinical signs of OPIDN.

Plasma ChE was strongly inhibited in the DFP-dosed birds (with or without PB) by approximately 90% in birds receiving 100 μg/kg DFP and 98% in birds dosed with 200 μg/kg DFP (Figure 19). ChE was depressed to this level after the initial dose and the depressed level was maintained throughout the period of treatment. Plasma ChE of birds receiving PB alone was initially inhibited 30%, with inhibition increasing to ~70% with subsequent carbamate dosing.

Clinical signs of OPIDN were observed in the treatment groups as follows: no signs were seen in the atropine controls; no signs were seen in animals treated with 100 μg/kg PB; birds in the 100 μg/kg DFP group first exhibited stage 2 at day 22 and day 28, and the third bird progressed to borderline stage 1/2 at day 34; the 200 μg/kg DFP birds reached stage 2 at day 17/18, 2 birds reached stage 3 at day 21/22 (then perfused); only 1 of the 100 μg/kg DFP + PB birds reached

stage 2, at day 37; the 200 μg/kg DFP + PB birds reached stage 2 at day 14/16 and stage 3 at day 17/21.

DFP-treated birds showing signs of OPIDN (stage 2 or 3) were perfused, along with atropine and PB controls. The 200 μg/kg DFP birds were perfused at 21/22 days, and the 100 μg/kg DFP birds at either 29 or 42 days. The perfused chickens were sent to OHSU for histological assessment (see Table 15). There was a general correlation between the OPIDN clinical stage observed (1 = normal to 5 = moribund) and the level of histological damage (0/1 = normal to 5 = advanced alterations, cellular destruction and cell loss).

Microscopical examination of sampled hind limb nerves of all animals treated with PB was within normal limits (Figure 20). DFP treatment was associated with distal nerve fiber damage in the absence (Figure 21A, 21C) and presence (Figure 21B, 21D) of concurrent PB treatment. In the absence of PB treatment, one animal (#30) showed no convincing changes, a second (#31) showed mild distal nerve changes, and a third (#43) showed changes distally and proximally (Table 15). Animals treated with 200 μg/kg DFP showed distal and proximal neuropathologic changes. However, one animal (#32) showed sural nerve damage while others (#'s 30 & 32) showed somewhat more advanced peripheral nerve pathology. There was some suggestion that PB enhanced hind limb nerve changes induced by DFP (Table 15).

DFP/PB Multiple Dosing Trial II

Results in previous experiments suggested that PB might have exacerbated damage in DFP-treated birds. This trial was conducted to address this question. One dose of DFP and three levels of PB were used (at one half to double the level used in the previous trial). Groups of chickens were treated with 200 μg/kg PB; 100 μg/kg DFP; 50 μg/kg PB + 100 μg/kg DFP; 100 μg/kg PB + 100 μg/kg DFP; 200 μg/kg PB + 100 μg/kg DFP (n = 2 for the PB control, n = 4 for all other groups). Treatments were given i.m in the pectoral muscle; PB was injected on the right side and then DFP was injected on the left side immediately afterward. The chickens were dosed 20 times over a one month period.

Chickens were observed for clinical signs of OPIDN for 3 weeks post-dosing. Final observations at sacrifice are summarized in Table 16. PB controls showed no clinical signs of OPIDN. The DFP controls had one bird with definite signs, 2 others with borderline signs and one with no signs. The groups of chickens that received DFP plus either the low (50 ug/kg) or high (200 ug/kg) PB treatment each had two of four birds with stage 2 signs and two birds with no signs. The DFP plus middle (100 ug/kg) PB treatment had all four birds with clinical signs of OPIDN: stage 2 or borderline stage 2/3.

The chickens were perfused at 49 days after the first dose and shipped to OHSU for histological assessment. Focus was on vulnerable distal regions of the tibial, peroneal and sural nerves. Results are summarized in Table 16.

XGB/PB Multiple Dosing Trial - Histological Endpoints

An experiment studying the effects of sarin and PB alone and in combination was conducted. Groups of chickens (n = 3) were dosed: atropine control (50 mg/kg); 100 μg/kg PB; 100 μg/kg DFP; 100 μg/kg PB + 100 μg/kg DFP; 12.5 μg/kg XGB; 100 μg/kg PB + 12.5 μg/kg XGB. All birds received atropine. Injections were given i.m. in the pectoral muscle; each agent was injected in a different area (upper or lower, right or left pectoral). Birds were dosed 5 days/week for 6 weeks. XGB (+/- PB)-treated birds and, less frequently, DFP-treated birds displayed mild signs of acute toxicity: head droop or drowsy appearance. Blood samples were taken periodically for plasma cholinesterase measurements. At the end of the dosing period, the birds were observed

for 3 weeks for clinical signs of OPIDN. Chickens were perfused and shipped to OHSU for histological assessment.

Early stages of OPIDN were observed in the DFP positive OPIDN controls (+/- PB) beginning 25-28 days after dosing began (first dose was on day 0). One bird, treated with DFP only, did not show signs until day 34. Dosing of these birds was stopped on day 25-29. All of the DFP (+/- PB) treated birds were perfused on day 41, along with one atropine control and one PB control. Clinical signs of OPIDN were observed in these birds as follows: the DFP-treated group had 2 birds at stage 3 and 1 bird at stage 2 (the bird that first showed signs at day 34); the DFP + PB treated birds had 2 birds at stage 3 and 1 bird at stage 4. The two controls were stage 1 (normal). No clinical signs of OPIDN were seen in any chickens that were not treated with DFP. The remaining birds were perfused on day 61/62.

Plasma ChE values are shown in Figure 22. The ATR control group had no ChE depression over the course of the experiment. The PB control group was inhibited 70%. All treatments that included OPs (DFP or XGB) inhibited plasma ChE 85% or more. ChE levels recovered when dosing was stopped: at week 4 for the DFP treatments; week 6 for the other treatments.

Figure 23 shows that the plasma ChE inhibition after the first treatment was consistent with the inhibition over the course of dosing. The amount of recovery in 24 hours, prior to the next treatment, ranged from 40% of initial activity in treatments including DFP, to 60% for XGB (+/- PB), to 75% for PB. ChE levels were unchanged in the ATR control group.

There was a loss of weight in all OP-treated animals during dosing. The XGB/PB treatment caused a 20% decrease in weight, and there was a 5% to 15% decrease in all other treatments. The DFP (+/- PB) treated chickens had continued weight loss after the dosing was ended at 4 weeks. This loss is consistent with evident OPIDN. All other animals showed an increase in weight after dosing ended at 6 weeks.

Results of histological examination of distal nerves are shown in Table 17. Sarin treatment with or without PB failed to induce OPIDN.

XGB/PB Multiple Dosing Trial - Biochemical Endpoints
Groups of chickens (n = 3) were treated as follows: atropine control (50 mg/kg); 100 μg/kg PB; 100 μg/kg DFP; 100 μg/kg PB + 100 μg/kg DFP; 12.5 μg/kg XGB; 100 μg/kg PB + 12.5 μg/kg XGB. All groups were treated with atropine. Birds received 30 doses daily (5 days/week) over a 42 day period. Mild acute toxicity signs were observed as in the histochemical trial above. Blood samples were taken periodically for cholinesterase (ChE) measurements. All birds were sacrificed on day 43. Brains were removed for measurement of AChE and NTE levels.

At sacrifice, only birds in the DFP group (2 of 3) and the PB + DFP group (2 of 3) were exhibiting clinical signs of OPIDN: all were stage 2. The early stages of OPIDN were observed beginning 33 days after dosing began (first dose was on day 0) in 2 birds (1 each in the DFP +/- PB groups). The other 2 birds first showed clinical signs on days 39 and 40. No clinical signs of OPIDN were seen in any chickens that were not treated with DFP.

Plasma ChE values are shown in Figure 24. Levels in the ATR control group declined by 30% over the course of treatment. The PB control group was inhibited 60%. Birds treated with DFP +/- PB had ChE levels inhibited by more than 90%. Plasma ChE in chickens treated with XGB+/- PB were inhibited 80%. ChE levels recovered by 40% in the PB and organophosphate treated birds at sacrifice (the day after dosing stopped).

There was a loss of weight in all treatments during dosing. The ATR and PB treated birds lost 10%. Treatment with OPs (+/- PB) caused a 15% to 25% decrease in weight.

Brain AChE levels are shown in Figure 25. PB treated birds showed no inhibition of AChE levels compared to the controls (ATR). Brain AChE activity in DFP (+/- PB)-treated chickens was inhibited 55%, and in XGB (+/- PB)-treated chickens was inhibited 70%.

Brain NTE levels are shown in Figure 26. PB treated birds showed no inhibition of NTE levels compared to the controls. Brain NTE activity in DFP (+/- PB)-treated chickens was inhibited over 90%, and in XGB (+/- PB) treated chickens was inhibited 35% and 45% (respectively).

KEY RESEARCH ACCOMPLISHMENTS

- Lethal dose levels of DFP and paraoxon were observed in mice, allowing dose limits to be set.
- IC_{50} values were determined for *in vitro* inhibition of AChE and NTE by sarin.
- IC_{50} values were determined for *in vitro* inhibition of AChE and NTE by PB.
- *In vitro* tests show no appreciable interaction of sarin and pyridostigmine in the inhibition of AChE and NTE.
- Lethal doses of PB inhibited brain AChE in hens, indicating that the active pyridostigmine anticholinesterase moiety of PB crosses the blood-brain barrier under these conditions.
- A single dose of TOCP near the no-effect level for OPIDN induction was determined in hens. For birds that exhibited clinical signs, the pattern and distribution of neuropathology matched that expected for OPIDN.
- Semi-quantifiable degrees of nerve fiber degeneration were demonstrable morphologically in hens with clinical evidence of TOCP-induced OPIDN. Myelinated nerve fibers in distal sural nerve were the first to be noted in the neurodegenerative process.
- Mice treated with single large doses of TOCP developed no clinical or pathological signs of OPIDN.
- PB did not inhibit brain NTE in treated birds, in agreement with our earlier *in vitro* findings.
- While sarin inhibited brain NTE *in vitro*, multiple doses (20 over 4 weeks) of sarin at the highest tolerated level (12.5 ug/kg) did not induce clinical or neuropathological signs of OPIDN in hens. PB treatment did not affect the inability of sarin to induce OPIDN under the conditions studied.
- PB treatment had no detectable effects on peripheral nerve fiber integrity.
- PB treatment given in conjunction with TOCP (before or after) had no observable effect on clinical signs of OPIDN.
- Concurrent treatment of hens with multiple doses of DFP + PB suggested a greater degree of nerve damage than in animals treated with DFP alone in some birds. This result should be confirmed.
- Multiple sub-lethal doses of PB in hens (30 over 6 weeks) did not cross the blood-brain barrier as evidenced by lack of depression of brain cholinesterase.

REPORTABLE OUTCOMES

Wilson BW, Henderson JD, Ramirez A, Kayton R and Spencer PS. 2002. Does pyridostigmine affect organophosphate induced nerve damage? Presented at Bioscience 2002 Medical Defense Review. Hunt Valley, Maryland, June 2-7.

Wilson BW, Henderson JD, Coatney EM, Nieberg PS and Spencer PS. 2002. Actions of pyridostigmine and organophosphate agents on chick cells, mice and chickens. Drug and Chemical Toxicology, 25(2), 131-139.

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CONCLUSIONS

There was no appreciable interaction *in vitro*, between sarin and pyridostigmine affecting the inhibition of AChE or NTE. This suggests that any potential effects of the combination we may find, would arise from interactions at the metabolic, cellular or higher levels.

A high level of NTE inhibition (>90%) was observed in sarin treated birds (3 doses of 250 ug/kg) without overt pathological signs of OPIDN. This suggests a dose at or near the No Effect Level for studies of sarin and pyridostigmine in combination. The 250 ug/kg dose level could not be tolerated for longer term experiments.

Multiple doses of sarin at high levels resulted in no clinical signs of OPIDN. The higher dose of 100 μg/kg proved lethal, even though atropine and 2-PAM treatment were used. The other 2 doses (25 and 50 μg/kg) were tolerated over a 4-week period with atropine treatment, but they caused strong acute symptoms and depressed plasma ChE levels by 80% or more. The sarin dose had to be reduced to 12.5 ug/kg in the longer term 6 week dosing trials.

One of the reasons PB was chosen as a nerve agent-antidote enhancer is that it is considered not to cross the blood-brain barrier and enter brain tissue. Since high doses of PB (≥2 mg/kg) in chickens inhibit brain AChE, the active pyridostigmine moiety evidently crossed the blood-brain

barrier under the conditions studied. Since sedatives based on bromides have well established CNS neurotoxic potential, the bromide moiety likely accompanies the pyridostigmine moiety across the blood-brain barrier. However, further studies using lower doses of PB are needed to investigate levels and conditions under which the barrier is breached (Friedman *et al.*, 1996).

The lack of brain AChE inhibition by sub-chronic PB treatment in chickens (100 ug/kg) indicates that PB did not cross the blood-brain barrier. This is in contrast to the high lethal acute doses of PB which did cross the barrier and inhibit AChE. The multiple dose treatment did inhibit plasma ChE levels by 60%, so the dose was at an effective level.

TOCP induces in chickens a reproducible avian model of OPIDN that can be scored in terms of severity by semi-quantitative light-microscopic analysis of cross-sections of distal peripheral nerves excised from animals perfused with chemical fixatives that preserve tissue ultrastructure. By contrast, PB did not produce neuropathological changes in this species. Chickens are therefore suitable to assess whether PB modulates the neuropathy-producing actions of TOCP provided that a dosage is selected in the mid-range of the OP's neurotoxic potential. Further studies with a larger number of animals and an optimum TOCP dosage (75 mg/kg s.c.) would be needed to confirm preliminary observations suggesting an absence of PB modulation of TOCP neurotoxicity.

A low-level of TOCP in hens that induces OPIDN was determined. Mice treated with 85x this level of TOCP did not develop signs of OPIDN.

Multiple dose levels of DFP that induce OPIDN in hens were established. The effect of PB exposures alone and in conjunction with DFP was studied. There was no clear-cut pattern of response that would allow one to conclude that PB modulates DFP-induced OPIDN. However, there was evidence to suggest that DFP + PB treatment (100/100 mg/kg) induced pathological changes in nerves that were not seen in animals treated with equivalent doses of DFP alone.

Sarin with or without PB-treatment failed to induce OPIDN. The sarin treatment was given at the highest dose, which could be tolerated without 2-PAM antidote over a 6 week period. There were no clinical or histological signs of OPIDN in birds, which received this treatment. This correlates with the observed level of brain NTE inhibition, which is lower than the 80% NTE inhibition threshold associated with the onset of OPIDN (Johnson, 1975).

The study was devised amidst concerns that the first Gulf War had generated a neuropathy based on multiple exposures to environmental chemicals (pesticides, repellants) used in the sector and PB. The fundamental paradigm was to use a minimal dose of a known organophosphate neuropathic chemical and the carbamate pyridostigmine bromide asking whether the combination would be more neurotoxic than either alone. The study demonstrated synergisms with regard to cholinesterase inhibitions but not consistently with regard to overt symptoms and morphological damage. One problem was inherent in the study design: low minimally neurotoxic doses are more likely to engender variable responses than levels higher up the dose response curve. Another was that levels of NTE inhibition that accompany OPIDN were not always as consistent in producing histological nerve damage as we expected. Indeed, Oliveira *et al.*, 2002 reported that 70-80% inhibition of NTE inhibition was insufficient to produce overt symptoms of OPIDN in three different strains of chickens.

Since the start of the research a number of papers have been published on the subject of delayed neuropathy. One was an interesting report of the development of a tyrosinase carbon paste electrode to detect NTE activity in whole blood (Makhaeva *et al.*, 2003). Another was a review

of Jamal (Jamal *et al.*, 2002) reiterating his position that "the weight of current evidence is...very much in favor of the notion that chronic low-level exposure to OP produces neurotoxicity". The results of this study do not support such a conclusion given the exposure scenarios studied. Jamal would not have agreed, saying that criticisms against his position "are unfounded and probably misconceived."

Taken at face value the data suggest that PB does not add to the neuropathic load of chemicals in a multiple exposure scenario. On the one hand, researchers find such negative evidence –of what did not happen-to be the bane of their existence. But, on the other hand, public health scientists are pleased to find scenarios suggesting the safe use of known neuroactive chemicals. In this case, the FDA has recently approved use of pyridostigmine bromide (30 mg tablets, three times a day) as a prophylactic for possible nerve gas exposures. To date, thankfully, there have been no published reports of a Gulf War II syndrome.

Regardless, research on the neurotoxic effects of multiple chemical exposures will continue as new information appears concerning the mechanisms of action of the chemicals themselves and the significance of the biomarkers used. For example, the recent report of Winrow *et al.* (2003) that knockout mice with disruptions to NTE (+/-) have lower brain NTE activity, higher mortality when exposed to an NTE inhibitor and hyper motor activity.

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APPENDICES

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Table 1. Tissues Sampled for Morphological Assessment

Frontal Cortex	Soleus Muscle
Basal Ganglia	Sural Nerve Proximal
Hippocampus	Sciatic Nerve Distal
Cerebellar Vermis	Diaphragm Muscle
Medulla Oblongata	Interosseous Muscle
Spinal Cord Cervical	Lumbrical Muscle
Spinal Cord Thoracic	Tibial Nerve Mid
Spinal Cord Lumbar	Tibial Nerve Distal
Lumbar Dorsal Root Ganglia	Sciatic Nerve Mid
Sciatic Nerve Proximal	Sural Nerve Mid
Sciatic Nerve Mid-thigh	Tibial Nerve Proximal
Gastrocnemius	Tibial Nerve at Ankle
Sural Nerve Distal	Peroneal Nerve Proximal
Peroneal Nerve Mid	Peroneal Nerve Distal

Table 2. Mouse Brain Enzyme Activity: Acute Dose

Dose Group	AChE	NTE
Saline	139 ± 21.4	17.8 ± 5.68
DFP (1.5 mg/kg)	22.5 ± 10.9	7.30 ± 2.09
Paraoxon (0.4 mg/kg)	34.3 ± 28.2	11.6 ± 10.2

Values are mean ± SD; n = 3; Activity is nmol/min/mg protein.
Dose administered s.c. in saline.

Table 3. Mouse Brain Enzyme Activity Following DFP Treatment

Group	AChE	NTE
Atropine Control	116 ± 19.2	16.6 ± 0.80
3 mg/kg DFP	22.8 ± 6.4	9.17 ± 3.45
4.5 mg/kg DFP	14.3 ± 9.0	2.52 ± 2.32
6 mg/kg DFP	8.8 ± 8.8	1.10 ± 1.68

Values are mean ± SD; n =3; Activity is nmol/min/mg protein.
Dose administered s.c. in saline.

Table 4. ChE Inhibition in DFP Treated Hens

	% Initial Plasma ChE activity			
	3-Nov	3-Nov	5-Nov	8-Nov
Band #	Predose	Postdose	Postdose	Postdose
14736	100.0%	-1.0%	0.9%	0.7%
14729	100.0%	1.6%	1.6%	3.5%
14251	100.0%	1.8%	2.7%	4.6%
14234	100.0%	14.2%	12.0%	14.9%
Mean	100.0%	4.2%	4.3%	5.9%
sd	0.0%	6.8%	5.2%	6.2%

Hens were dosed i.m. with 300 ug/kg DFP in saline.
Postdose blood samples were taken 30 min after dosing.

Table 5. ChE Inhibition in PB Dosed Hens

		% Plasma ChE activity		
PB Dose	Band #	Predose	1 Hr	24 Hr
0	14769	100.0%	88.0%	110.0%
0	14773	100.0%	108.1%	116.0%
1	14202	100.0%	32.5%	70.4%
1	14206	100.0%	35.3%	73.1%
2	14771	100.0%	-2.3%	---
2	14766	100.0%	-6.9%	58.3%
5	14770	100.0%	---	---
5	14764	100.0%	---	---
10	14204	100.0%	---	---
10	14250	100.0%	---	---

PB dose (mg/kg) administered i.m. in water.
--- No value; bird died.

Table 6. Morphological Assessment of TOCP Treated Hens

Animal #	TOCP Dose	Tissue	Score
14207	0	PD	1
		SUD	0
		TD	0
14244	0	PD	0
		SUD	0
		TD	0
14733	50	PD	0
		SUD	1
		TD	1
14223	50	**PD**	**2**
		SUD	**3**
		TD	1
14225	100	**PD**	**2.5**
		SUD	**3**
		TD	**2.5**
14261	100	**PD**	**2**
		SUD	**3**
		TD	**3**
14237	150	**PD**	**2**
		SUD	**2.5**
		TD	**2**
14774	150	**PD**	**2.5**
		SUD	**3**
		TD	**2**

TOCP dose (mg/kg) administered s.c. (neat).
Tissues examined include Peroneal Nerve Distal (PD), Sural Nerve Distal (SUD) and Tibial Nerve Distal (TD).
Bold entries indicate significant pathology.

Table 7. Morphological Assessment of Hens Treated with TOCP

Animal	TOCP Dose	OPIDN Sign at Perfusion	Histological Score	Examined Tissue
19	0 mg/kg	1	1	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			1	Sural Nerve Distal
			1	Sural Nerve Proximal
			1	Tibial Nerve Distal
			1	Tibial Nerve Proximal
			2	Spinal Cord Cervical
			1	Spinal Cord Lumbar
			1	Medulla Oblongata
22	50 mg/kg	1	1	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
25	50 mg/kg	1	1	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			1	Sural Nerve Proximal
			1	Tibial Nerve Distal
			1	Tibial Nerve Proximal
			1	Spinal Cord Cervical
			2	Medulla Oblongata
17	75 mg/kg	1	1	Peroneal Nerve Distal
			1	Sural Nerve Distal
			0	Tibial Nerve Distal
18	75 mg/kg	3/4	3	Peroneal Nerve Distal
			3	Peroneal Nerve Proximal
			3	Sural Nerve Distal
			3	Sural Nerve Proximal
			3	Tibial Nerve Distal
			3	Tibial Nerve Proximal
			3	Spinal Cord Cervical
			2	Spinal Cord Lumbar
			1	Sciatic Nerve Proximal
			2	Medulla Oblongata

Table 7. Morphological Assessment of Hens Treated with TOCP (Cont.)

Animal	TOCP Dose	OPIDN Sign at Perfusion	Histological Score	Examined Tissue
20	75 mg/kg	3	**3**	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			4	Sural Nerve Proximal
			2	Tibial Nerve Distal
			3	Tibial Nerve Proximal
			2	Spinal Cord Cervical
			2	Spinal Cord Lumbar
			1	Sciatic Nerve Proximal
			2	Medulla Oblongata
23	100 mg/kg	3	**3**	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			4	Sural Nerve Distal
			3	Sural Nerve Proximal
			2	Tibial Nerve Distal
			4	Tibial Nerve Proximal
			2	Spinal Cord Cervical
			1	Spinal Cord Lumbar
			1	Sciatic Nerve Proximal
			2	Medulla Oblongata
24	100 mg/kg	3	**3**	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			5	Sural Nerve Distal
			3	Sural Nerve Proximal
			2	Tibial Nerve Distal
			3	Spinal Cord Cervical
			2	Spinal Cord Lumbar
			1	Sciatic Nerve Proximal
			3	Medulla Oblongata
			3	Tibial Nerve Proximal

TOCP dose (mg/kg) administered s.c. (neat).
Bold entries indicate significant pathology.

Table 8. Morphological Assessment of Mice Treated with TOCP

Animal	TOCP Dose	Histological Score	Examined Tissue
5A	0 mg/kg	1	Peroneal Nerve Distal
		1	Sciatic Nerve Distal
		0	Sural Nerve Distal
		1	Tibial Nerve Distal
5B	0 mg/kg	0	Peroneal Nerve Distal
		1	Sural Nerve Distal
		2	Tibial Nerve Distal
6A	0.8mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
6B	0.8mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		0	Tibial Nerve Distal
7A	1.6 g/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
7B	1.6 g/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
8B	3.2 g/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal

TOCP dose (mg/kg) administered s.c. in corn oil.

Table 9. Morphological Assessment of TOCP-Treated Mice

Animal	TOCP Dose	Histological Score	Examined Tissue
15	0 mg/kg	0	Sural Nerve Distal
		1	Tibial Nerve Distal
16	0 mg/kg	0	Peroneal Nerve Distal
		0	Spinal Cord Cervial
		1	Tibial Nerve Distal
13	3.2 g/kg	0	Peroneal Nerve Distal
		0	Sural Nerve Distal
		1	Tibial Nerve Distal
14	3.2 g/kg	1	Peroneal Nerve Distal
		0	Sural Nerve Distal
		0	Tibial Nerve Distal
11	6.4 g/kg	2	Peroneal Nerve Medial
		0	Sural Nerve Distal
		2	Tibial Nerve Distal
12	6.4 g/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
		1	Medulla Oblongata

TOCP dose (mg/kg) administered s.c. (neat).

Table 10. Enzyme Inhibitions in Chickens Given Multiple Treatments

Group	Dose	Initial Blood ChE Inhibition	Brain AChE		Brain NTE	
			Act	% Inh	Act	% Inh
XGB	250 ug/kg	41%	16.9	86%	1.02	92%
DFP	200 ug/kg	94%	47.4	61%	2.43	80%
PO	200 ug/kg	77%	26.4	78%	11.4	6%
ATR	20 mg/kg	4%	121	0%	12.0	0%

Initial Blood ChE Inhibition is ~1 hr post-dose. Dose administered i.v. in saline.
Brain: Act is activity in nmol/min/mg; % Inh is inhibition compared to ATR.

Table 11. Blood ChE Inhibition in Chickens Dosed for OPIDN Trial

Group	Dose	ChE Inhibition		
		1st Dose	2nd Dose	3rd Dose
XGB	250 ug/kg	48%	54%	---
DFP	200 ug/kg	94%	95%	92%
PO	200 ug/kg	64%	86%	87%
ATR	20 mg/kg	0%	(-)4%	(-)5%
XGB	200/150 ug/kg	54%	48%	40%

Blood ChE Inhibition is ~1 hr post-dose; --- no value, birds died.

Table 12. Morphological Assessment of OP-Treated Birds

Dose	Animal	Score	Tissue
Atropine Control	14716	1	Peroneal Nerve Distal
		3	Sural Nerve Distal
		2	Spinal Cord Lumbar
		1.5	Tibial Nerve Distal
Atropine Control	14707	2	Peroneal Nerve Distal
		1	Sural Nerve Distal
		2	Spinal Cord Lumbar
		0.5	Tibial Nerve Distal
Atropine Control	14740	2	Peroneal Nerve Distal
		2.25	Sural Nerve Distal
		1	Spinal Cord Lumbar
		2.25	Tibial Nerve Distal
200 ug/kg Paraoxon	14702	0	Peroneal Nerve Distal
		2	Spinal Cord Lumbar
		0	Tibial Nerve Distal
200 ug/kg Paraoxon	14738	1.5	Peroneal Nerve Distal
		1.5	Sural Nerve Distal
		2.25	Spinal Cord Lumbar
		2	Tibial Nerve Distal
200 ug/kg DFP	14710	1	Peroneal Nerve Distal
		0.5	Sural Nerve Distal
		2	Spinal Cord Lumbar
		2	Tibial Nerve Distal
200 ug/kg DFP	14725	1.5	Sural Nerve Distal
		3.25	Spinal Cord Lumbar
		1	Tibial Nerve Distal
200/150 ug/kg XGB	14711	0.5	Peroneal Nerve Distal
		1	Sural Nerve Distal
		2.5	Spinal Cord Lumbar
		1	Tibial Nerve Distal
200/150 ug/kg XGB	14734	1	Peroneal Nerve Distal
		0	Sural Nerve Distal
		2.25	Spinal Cord Lumbar
		1.5	Tibial Nerve Distal

Table 13. Morphological Assessment of Hens Treated with Multiple Doses of Sarin

Animal	Treatment	Score	Tissue
1	Sarin 25 ug/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		0	Tibial Nerve Distal
2	Sarin 25 ug/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
3	Sarin 50 ug/kg	0	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
4	Sarin 50 ug/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1.5	Tibial Nerve Distal
7	Atropine 20 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
8	Atropine 20 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
9	PB 0.1 mg/kg	**5**	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
10	PB 0.1 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
11	PB 0.25 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
12	PB 0.25 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
13	PB 0.5 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
14	PB 0.5 mg/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
15	DFP 100 ug/kg	1	Peroneal Nerve Distal
		1	Sural Nerve Distal
		1	Tibial Nerve Distal
16	DFP 100 ug/kg	**2**	Peroneal Nerve Distal
		2	Sural Nerve Distal
		4	Tibial Nerve Distal

Table 14. Morphological Assessment of Hens Dosed with TOCP and PB

Animal #	Dose Group	OPIDN Clinical Stage	Examined Tissue	Morphological Score
14221	PB	1	Peroneal Nerve Distal	1.5
			Sural Nerve Distal	0.5
			Tibial Nerve Distal	1.5
14703	PB	1	Peroneal Nerve Distal	1
			Sural Nerve Distal	1
			Tibial Nerve Distal	0.5
14755	PB	1	Peroneal Nerve Distal	1
			Sural Nerve Distal	0
			Tibial Nerve Distal	1
14772	TOCP	3/4	Peroneal Nerve Distal	**2**
			Sural Nerve Distal	**4**
			Tibial Nerve Distal	0.5
14242	TOCP	3/4	Peroneal Nerve Distal	**2.5**
			Sural Nerve Distal	**3.75**
			Tibial Nerve Distal	**2**
14767	TOCP	2	Peroneal Nerve Distal	0
			Sural Nerve Distal	**2**
			Tibial Nerve Distal	1.5
No Band	PB/TOCP	3	Peroneal Nerve Distal	1.5
			Sural Nerve Distal	**3**
14208	PB/TOCP	3	Peroneal Nerve Distal	**2.25**
			Sural Nerve Distal	**2.75**
			Tibial Nerve Distal	**2**
14762	PB/TOCP	3/4	Peroneal Nerve Distal	**3.5**
			Tibial Nerve Distal	**3**
14255	TOCP/PB	3/4	Peroneal Nerve Distal	**2.5**
			Sural Nerve Distal	**3.75**
			Tibial Nerve Distal	**3.5**
14232	TOCP/PB	3	Peroneal Nerve Distal	**2.5**
			Sural Nerve Distal	**4**
			Tibial Nerve Distal	**2**
14777	TOCP/PB	3/4	Peroneal Nerve Distal	**2.5**
			Sural Nerve Distal	**3**
			Tibial Nerve Distal	**3.75**

Dose groups: PB (0.5 mg/kg); TOCP (200 mg/kg); PB/TOCP (0.5 mg/kg PB followed 4 hrs later with 200 mg/kg TOCP); TOCP/PB (200 mg/kg TOCP followed 22 hrs later by 0.5 mg/kg PB).

Bold entries indicate significant pathology.

Table 15. Morphological Assessment of Hens Treated with DFP +/- PB (Trial I)

Animal	Dose Group	OPIDN Sign at Perfusion	Histological Score	Examined Tissue
26	Atropine	1	1	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			1	Tibial Nerve Distal
27	Atropine	1	0	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
			1	Tibial Nerve Proximal
39	Atropine	1	2	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
28	PB	1	1	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
29	PB	1	0	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
			1	Tibial Nerve Proximal
42	PB	1	1	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
			1	Tibial Nerve Proximal
30	DFP (100)	2	2	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			1	Lumbar Dorsal Root Ganglia
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
31	DFP (100)	1.5	2	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			1	Lumbar Dorsal Root Ganglia
			2	Sural Nerve Distal
			1	Sural Nerve Proximal
			2	Tibial Nerve Distal
			1	Tibial Nerve Proximal
43	DFP (100)	2	2	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			2	Sural Nerve Proximal
			2.5	Tibial Nerve Distal
			3	Tibial Nerve Proximal

Table 15. Morphological Assessment of Hens Treated with DFP +/- PB (Trial I; Cont.)

Animal	Dose Group	OPIDN Sign at Perfusion	Histological Score	Examined Tissue
32	DFP (200)	2	2	Peroneal Nerve Distal
			1	Peroneal Nerve Proximal
			1.5	Sural Nerve Distal
			4	Sural Nerve Proximal
			1	Tibial Nerve Distal
			2	Tibial Nerve Proximal
			1	Sciatic Nerve Proximal
33	DFP (200)	3	3	Peroneal Nerve Distal
			3	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			2	Sural Nerve Proximal
			2.5	Tibial Nerve Distal
			4	Tibial Nerve Proximal
44	DFP (200)	3	2	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			4	Sural Nerve Proximal
			2	Tibial Nerve Distal
			3	Tibial Nerve Proximal
34	DFP (100)/PB	2.5	3	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			3	Sural Nerve Distal
			3	Sural Nerve Proximal
			2	Tibial Nerve Distal
			2	Tibial Nerve Proximal
35	DFP (100)/PB	1	2	Peroneal Nerve Distal
			1.5	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			1	Sural Nerve Proximal
			2	Tibial Nerve Distal
			1.5	Tibial Nerve Proximal
45	DFP (100)/PB	1	1	Peroneal Nerve Distal
			1	Sural Nerve Distal
			1	Tibial Nerve Distal
36	DFP (200)/PB	3	3	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			4	Sural Nerve Proximal
			4	Tibial Nerve Distal
			4	Tibial Nerve Proximal

Table 15. Morphological Assessment of Hens Treated with DFP +/- PB (Trial I; Cont.)

Animal	Dose Group	OPIDN Sign at Perfusion	Histological Score	Examined Tissue
38	DFP (200)/PB	3	3	Peroneal Nerve Distal
			2	Peroneal Nerve Proximal
			2	Sural Nerve Distal
			4	Sural Nerve Proximal
			3	Tibial Nerve Distal
			4	Tibial Nerve Proximal
46	DFP (200)/PB	3	1	Peroneal Nerve Distal
			2	Sural Nerve Distal
			1	Tibial Nerve Distal

PB dose is 100 ug/kg; DFP dose is number in parentheses, in ug/kg.

TABLE 16. Morphological Assessment of Hens Treated with DFP +/- PB (Trial II)

Treatment	Dose	Hen #	OPIDN Score	TISSUE	SCORE
PB	200 ug/kg	4234	1	Peroneal Nerve Distal-Left	0
				Tibial Nerve Distal-Left	0
				Sural Nerve Distal-Left	1
				Peroneal Nerve Distal-Right	0
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	0
PB	200 ug/kg	4299	1	Peroneal Nerve Distal-Left	1
				Tibial Nerve Distal-Left	1
				Sural Nerve Distal-Left	1
				Peroneal Nerve Distal-Right	1
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	0
DFP	100 ug/kg	4210	2/3	Peroneal Nerve Distal-Left	2
				Sural Nerve Distal-Left	2
				Peroneal Nerve Distal-Right	1
				Tibial Nerve Distal-Right	2
				Sural Nerve Distal-Right	2
DFP	100 ug/kg	4293	½	Peroneal Nerve Distal-Left	1
				Tibial Nerve Distal-Left	1
				Sural Nerve Distal-Left	1
				Peroneal Nerve Distal-Right	1
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	1
DFP	100 ug/kg	4217	1	Peroneal Nerve Distal-Left	1
				Tibial Nerve Distal-Left	0
				Sural Nerve Distal-Left	0
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	0
DFP	100 ug/kg	4226	½	Peroneal Nerve Distal-Left	0
				Tibial Nerve Distal-Left	0
				Sural Nerve Distal-Left	0
				Peroneal Nerve Distal-Right	0
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	0
PB + DFP	50/100 ug/kg	4209	2	Peroneal N. Peripheral-Left	0
				Tibial Nerve Medial-Left	1
				Sural Nerve Distal-Left	0
				Peroneal Nerve Distal-Right	1
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	1

TABLE 16. Morphological Assessment of Hens Treated with DFP +/- PB (Trial II; Cont.)

Treatment	Dose	Hen #	OPIDN Score	TISSUE	SCORE
PB + DFP	50/100 ug/kg	4233	1	Peroneal Nerve Distal-Left	1
				Tibial Nerve Distal-Left	0
				Sural Nerve Distal-Left	0
				Peroneal Nerve Distal-Right	0
				Tibial Nerve Distal-Right	0
				Sural Nerve Distal-Right	0
PB + DFP	50/100 ug/kg	4303	2	Peroneal Nerve Distal-Left	1
				Tibial Nerve Distal-Left	**2**
				Sural Nerve Distal-Left	1
				Peroneal Nerve Distal-Right	1
				Tibial Nerve Distal-Right	0
				Sural Nerve Distal-Right	**2**
PB + DFP	50/100 ug/kg	4304	1	Peroneal Nerve Distal-Left	1
				Tibial Nerve Distal-Left	1
				Sural Nerve Distal-Left	0
				Peroneal Nerve Distal-Right	1
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	1
PB + DFP	100/100 ug/kg	71	2	Peroneal Nerve Distal-Left	**2**
				Tibial Nerve Distal-Left	1
				Sural Nerve Distal-Left	**3**
				Peroneal Nerve Distal-Right	0
				Tibial Nerve Distal-Right	**2**
				Sural Nerve Distal-Right	**2**
PB + DFP	100/100 ug/kg	4212	2	Tibial Nerve Distal-Left	1
				Sural Nerve Distal-Right	0
				Peroneal Nerve Distal-Right	0
				Sural Nerve Distal-Right	0
PB + DFP	100/100 ug/kg	4230	2/3	Peroneal Nerve Distal-Left	1
				Peroneal Nerve Distal-Right *	1
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	1
PB + DFP	100/100 ug/kg	4256	2	Peroneal Nerve Distal-Left	1
				Tibial Nerve Distal-Left	0
				Sural Nerve Distal-Left	**3**
				Peroneal Nerve Distal-Right	1
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	**3**

TABLE 16. Morphological Assessment of Hens Treated with DFP +/- PB (Trial II; Cont.)

Treatment	Dose	Hen #	OPIDN Score	TISSUE	SCORE
PB + DFP	200/100 ug/kg	4292	1	Peroneal Nerve Distal-Left	1
				Tibial Nerve Distal-Left	0
				Sural Nerve Distal-Left	1
				Peroneal Nerve Distal-Right	1
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	1
PB + DFP	200/100 ug/kg	4227	1	Peroneal Nerve Distal-Left	1
				Tibial Nerve Distal-Left	**2**
				Sural Nerve Distal-Left	0
				Peroneal Nerve Distal-Right	0
				Tibial Nerve Distal-Right	0
				Sural Nerve Distal-Right	1
PB + DFP	200/100 ug/kg	4268	1	Peroneal Nerve Distal-Left	0
				Tibial Nerve Distal-Left	1
				Sural Nerve Distal-Left	**3**
				Peroneal Nerve Distal-Right	1
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	1
PB + DFP	200/100 ug/kg	4290	2	Peroneal Nerve Distal-Left	0
				Tibial Nerve Distal-Left	0
				Sural Nerve Distal-Left	1
				Peroneal Nerve Distal-Right	1
				Tibial Nerve Distal-Right	1
				Sural Nerve Distal-Right	1

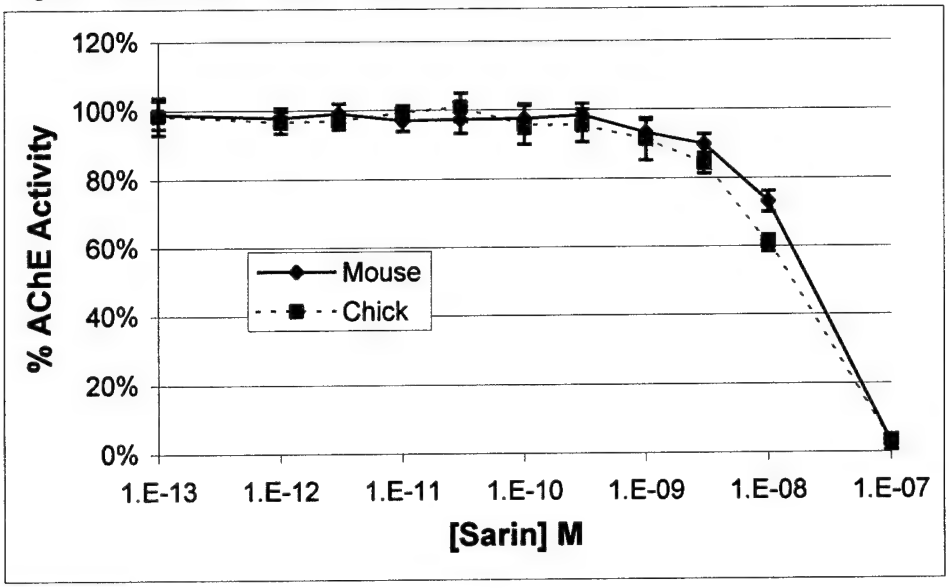
Table 17. Morphological Assessment of Hens Treated with Multiple Doses of XGB +/- PB

Treatment	Dose	Hen #	OPIDN Score	Tissue	Morphological Score
Atropine	50 mg/kg	47	1	Peroneal Nerve Distal	1
				Sural Nerve Distal	1
				Tibial Nerve Distal	1
Atropine	50 mg/kg	51	1	Peroneal Nerve Distal	1
				Sural Nerve Distal	0
				Tibial Nerve Distal	1
Atropine	50 mg/kg	63	1	Peroneal Nerve Distal	1
				Sural Nerve Distal	1
				Sural Nerve Proximal	1
				Tibial Nerve Distal	1
PB	100 ug/kg	52	1	Peroneal Nerve Distal	1
				Sural Nerve Distal	0
				Tibial Nerve Distal	0
PB	100 ug/kg	58	1	Peroneal Nerve Distal	0
				Sural Nerve Distal	1
				Tibial Nerve Distal	1
PB	100 ug/kg	64	1	Peroneal Nerve Distal	1
				Sural Nerve Distal	1
				Tibial Nerve Distal	1
DFP	100 ug/kg	53	3	Peroneal Nerve Distal	2
				Sural Nerve Distal	2
				Tibial Nerve Distal	2
DFP	100 ug/kg	59	2	Peroneal Nerve Distal	1
				Peroneal Nerve Medial	1
				Sural Nerve Distal	1
				Tibial Nerve Distal	1
DFP	100 ug/kg	65	3	Peroneal Nerve Distal	1
				Sural Nerve Distal	1
				Tibial Nerve Distal	1
DFP/PB	100/100 ug/kg	48	3	Peroneal Nerve Distal	3
				Sural Nerve Distal	0
				Tibial Nerve Distal	2
				Tibial Nerve Proximal	3
DFP/PB	100/100 ug/kg	54	4	Peroneal Nerve Distal	3
				Peroneal Nerve Proximal	0
				Sural Nerve Distal	1
				Sural Nerve Proximal	3
				Tibial Nerve Distal	2
				Tibial Nerve Proximal	3

Table 17. Morphological Assessment of Hens Treated with Multiple Doses of XGB +/- PB (Cont.)

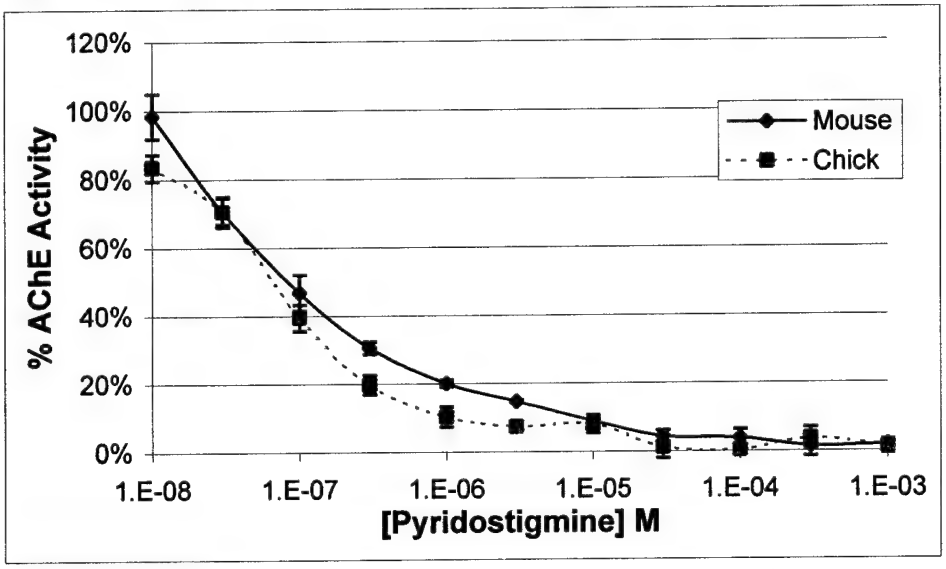
Treatment	Dose	Hen #	OPIDN Score	Tissue	Morphological Score
DFP/PB	100/100 ug/kg	66	3	Peroneal Nerve Distal	2
				Sural Nerve Distal	1-2
				Tibial Nerve Distal	2-3
XGB	12.5 ug/kg	55	1	Peroneal Nerve Distal	0
				Sural Nerve Distal	0
				Tibial Nerve Distal	0
XGB	12.5 ug/kg	61	1	Peroneal Nerve Distal	1
				Sural Nerve Distal	1
				Tibial Nerve Distal	1
XGB	12.5 ug/kg	67	1	Peroneal Nerve Distal	1
				Sural Nerve Distal	1
				Tibial Nerve Distal	2
XGB/PB	12.5/100 ug/kg	49	1	Peroneal Nerve Distal	1
				Spinal Cord Thoracic	0
				Tibial Nerve Distal	0
XGB/PB	12.5/100 ug/kg	56	1	Peroneal Nerve Distal	2
				Sural Nerve Distal	1
				Tibial Nerve Distal	1
XGB/PB	12.5/100 ug/kg	69	1	Peroneal Nerve Distal	1
				Sural Nerve Distal	2
				Tibial Nerve Distal	1

Figure 1. Inhibition of Brain AChE by Sarin



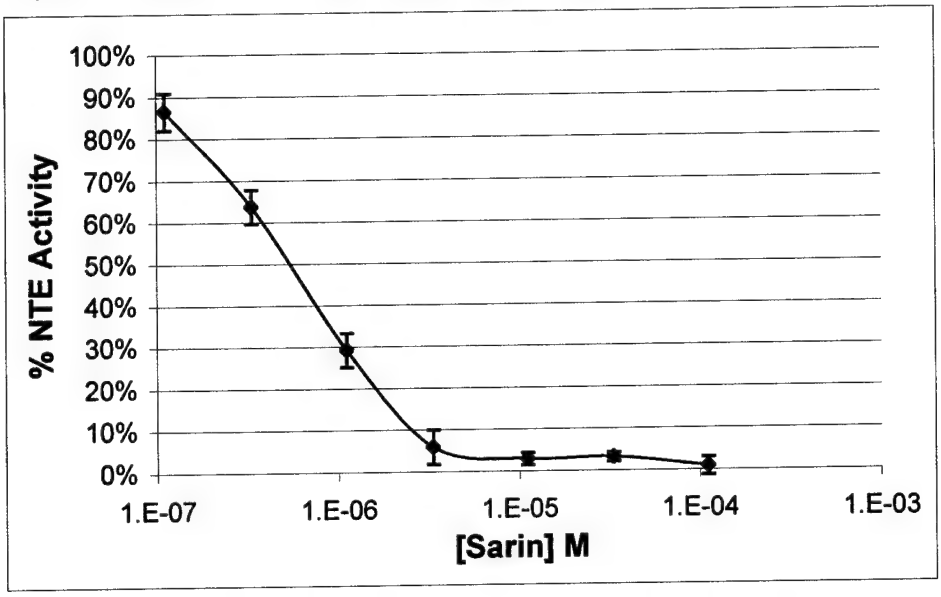
In vitro inhibition of brain AChE by sarin. Samples are assayed in triplicate. IC_{50} value is 4.0×10^{-8} M for mouse and 2.7×10^{-8} M for chicken.

Figure 2. Inhibition of Brain AChE by Pyridostigmine Bromide



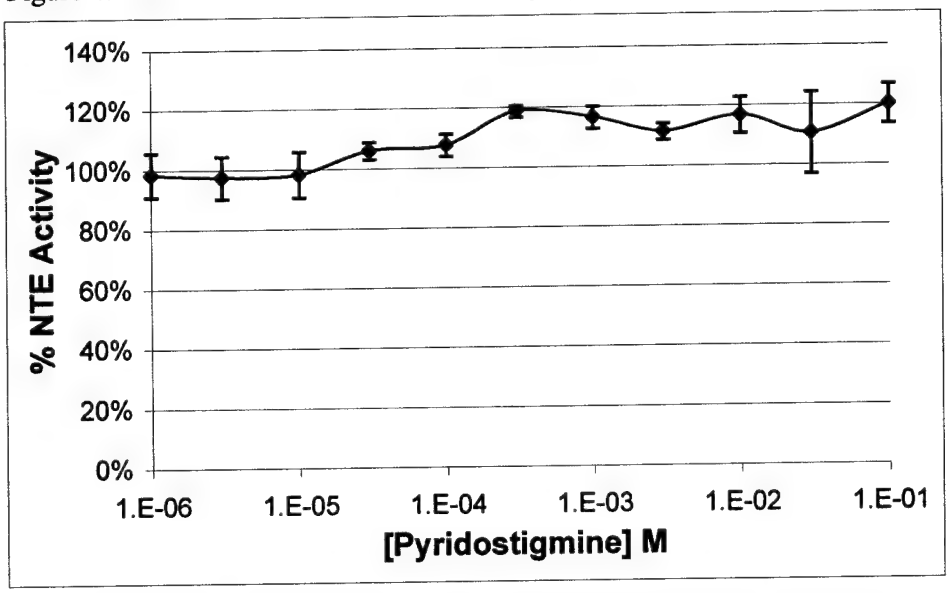
In vitro inhibition of brain AChE by pyridostigmine. Samples are assayed in triplicate. IC_{50} value is 9.1×10^{-8} M for mouse and 7.6×10^{-8} M for chicken.

Figure 3. Inhibition of Chicken Brain NTE by Sarin



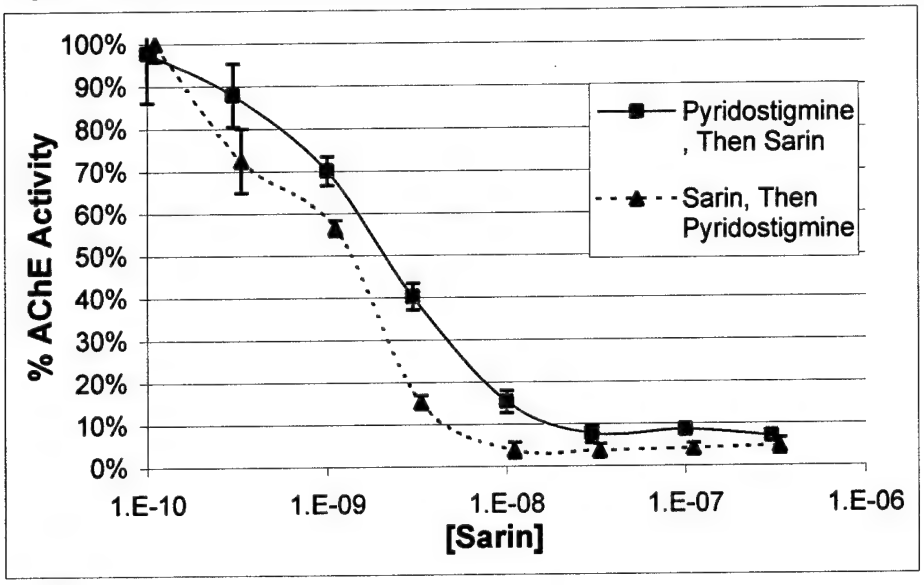
In vitro inhibition of brain NTE by sarin. Samples are assayed in triplicate. IC_{50} value is 6.4×10^{-7} M.

Figure 4. Inhibition of Chicken Brain NTE by Pyridostigmine Bromide



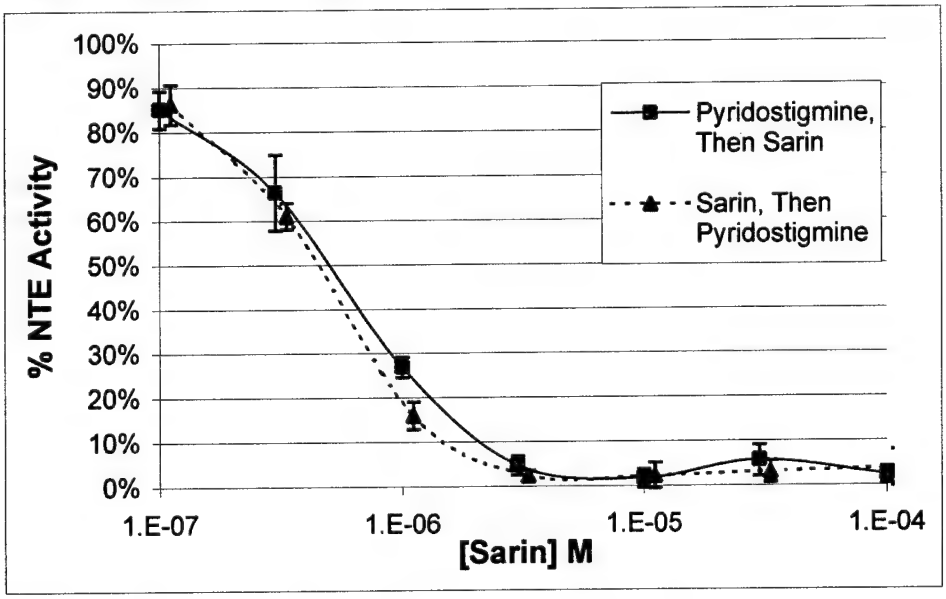
In vitro inhibition of brain NTE by sarin. Samples are assayed in triplicate. No inhibition was observed.

Figure 5. Inhibition of Mouse Brain AChE by Sarin and Pyridostigmine Bromide



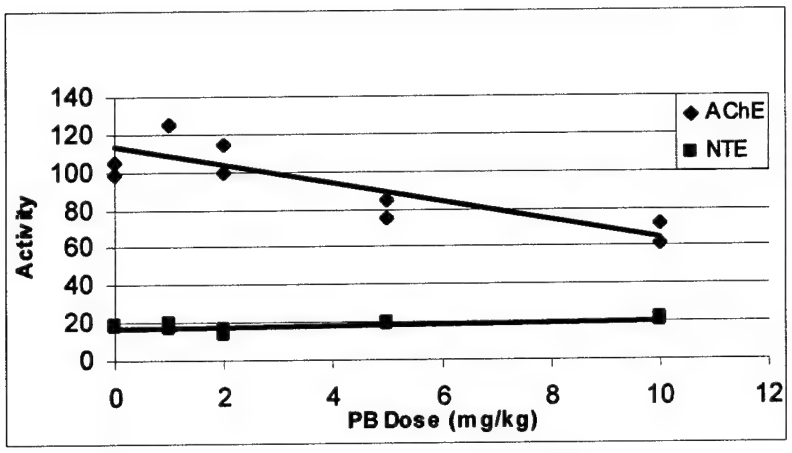
In vitro inhibition of brain AChE by sarin plus 3 x 10^{-8} M pyridostigmine.
Samples are assayed in triplicate. Pyridostigmine inhibits 70% of brain AChE.
Pattern of inhibition is the same for sarin alone.

Figure 6. Inhibition of Chicken Brain NTE by Sarin and Pyridostigmine Bromide



In vitro inhibition of brain NTE by sarin plus 1 x 10^{-2} M pyridostigmine.
Samples are assayed in triplicate. Pyridostigmine had no effect on NTE inhibition.

Figure 7. Brain Enzyme Levels in Hens Dosed with PB



Enzyme activities are nmol/min/mg protein.

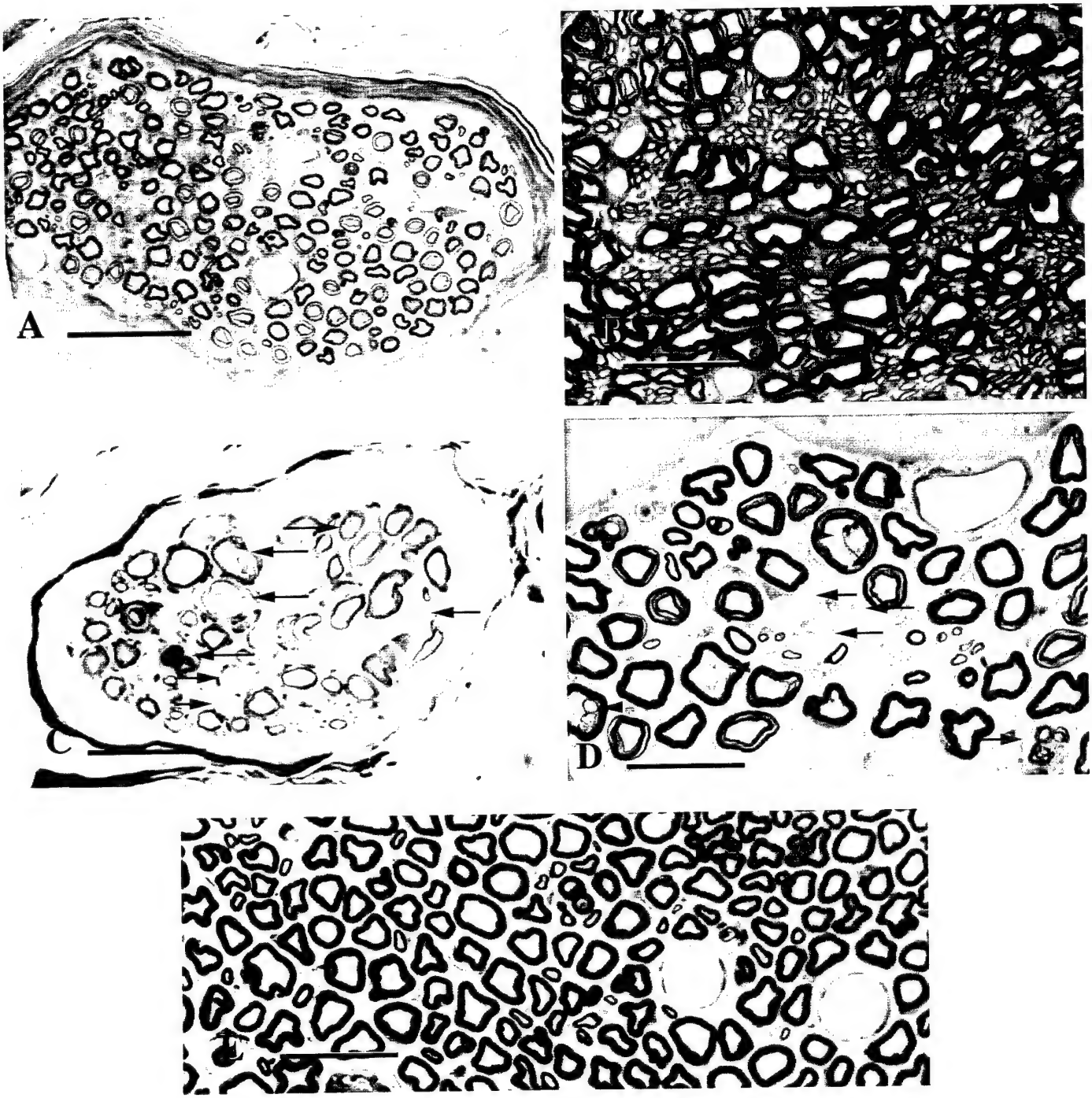


Figure 8. **A**, **B**, **C**, and **D** are Distal sural nerves in cross section from hens taken from the same level (ankle), **E**, is a cross section of the sciatic nerve at the level of the knee. **A** (hen # 14223)(bar = 66 μm) & **B** (hen #14773) (bar = 35 μm) demonstrate the varied effect of dosing at 50 mg/kg TOCP. While there are degenerating single large-diameter myelinated nerve fibers delimited by basal lamina (arrows) in **A**, **B** (at the same dose) shows no obvious neuropathology. **C**. Distal sural nerve in cross section from hen #14225 dosed at 100 mg/kg TOCP showing degenerating single large-diameter myelinated nerve fibers delimited by basal lamina (arrows) (bar = 47 μm). **D**. Similar neuropathy is seen when the dose is 150 mg/kg TOCP (arrows) hen #14774, also seen is evidence of primary axonal degeneration (arrowhead) characterized by large number of vesicular structures within the axon(bar = 28 μm). **E**. Distal sciatic nerve at knee in cross section (no band) dosed at 200mg/kg PB/TOCP. This proximal region is normal in appearance while the distal to this we see stage 2, 3, & 4 degeneration at this dose (bar = 31 μm).

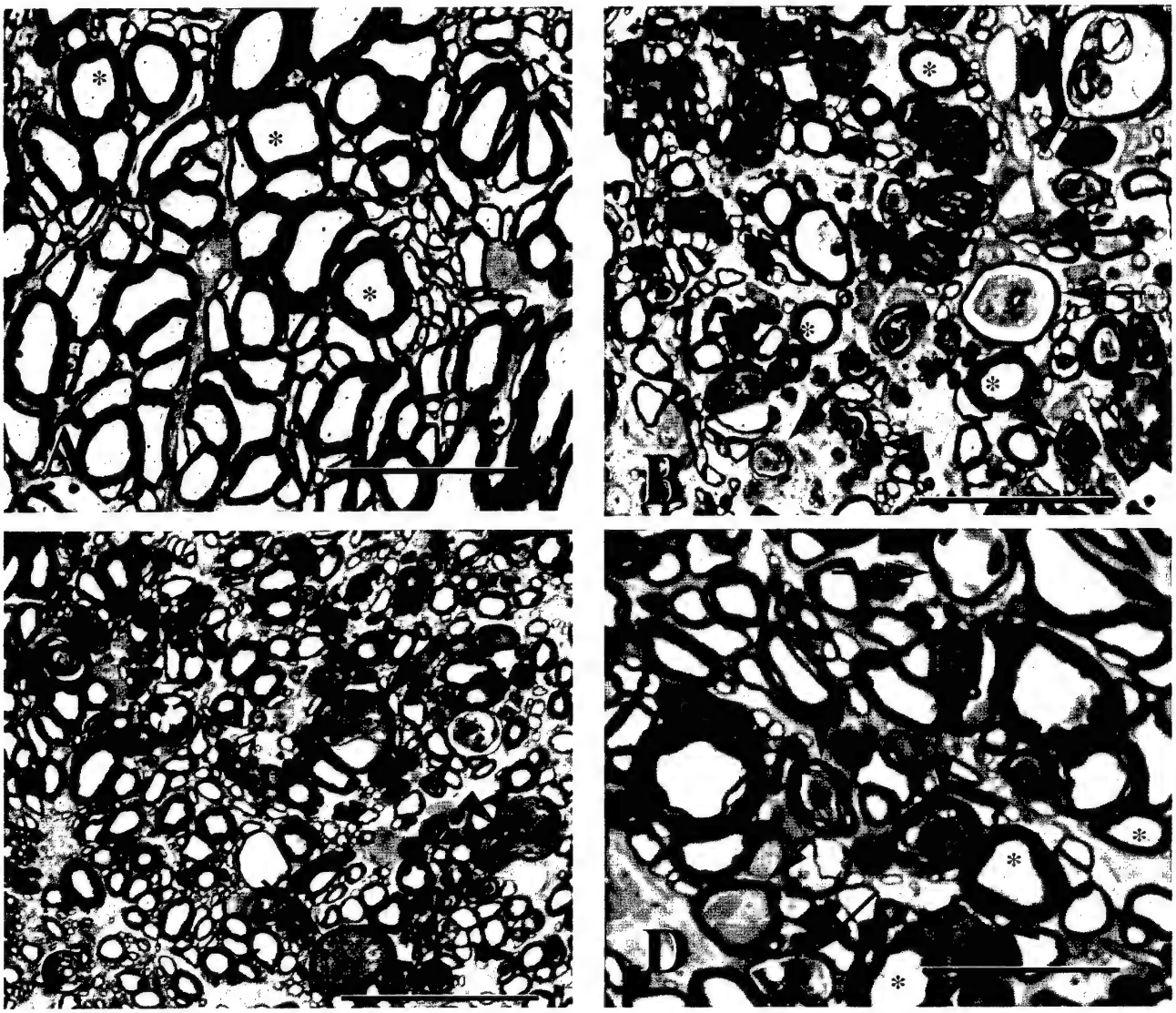


Figure 9. Cross sections of hen medulla oblongata showing areas of myelinated nerve fibers in tracts corresponding to the distal ends of ascending spinal pathways. **A.** #19 (control), showing normal morphology (stage 0-1) (bar = 40 μm). **B.** #18 (75 mg/kg TOCP) showing extensive myelinated nerve fiber degeneration (arrows). Arrows denote stages in axonal degeneration and asterisks are intact fibers (stage 2) (bar = 44 μm). **C.** #24 (100 mg/kg TOCP) showing similar stages of degeneration as B (arrows) (stage 3) (bar = 56 μm). **D.** #23 (100 mg/kg TOPC) demonstrating primary axonal degeneration (arrows) . Note myelinated nerve fibers that are normal in appearance (asterisks) (stage 2) (bar = 20 μm).

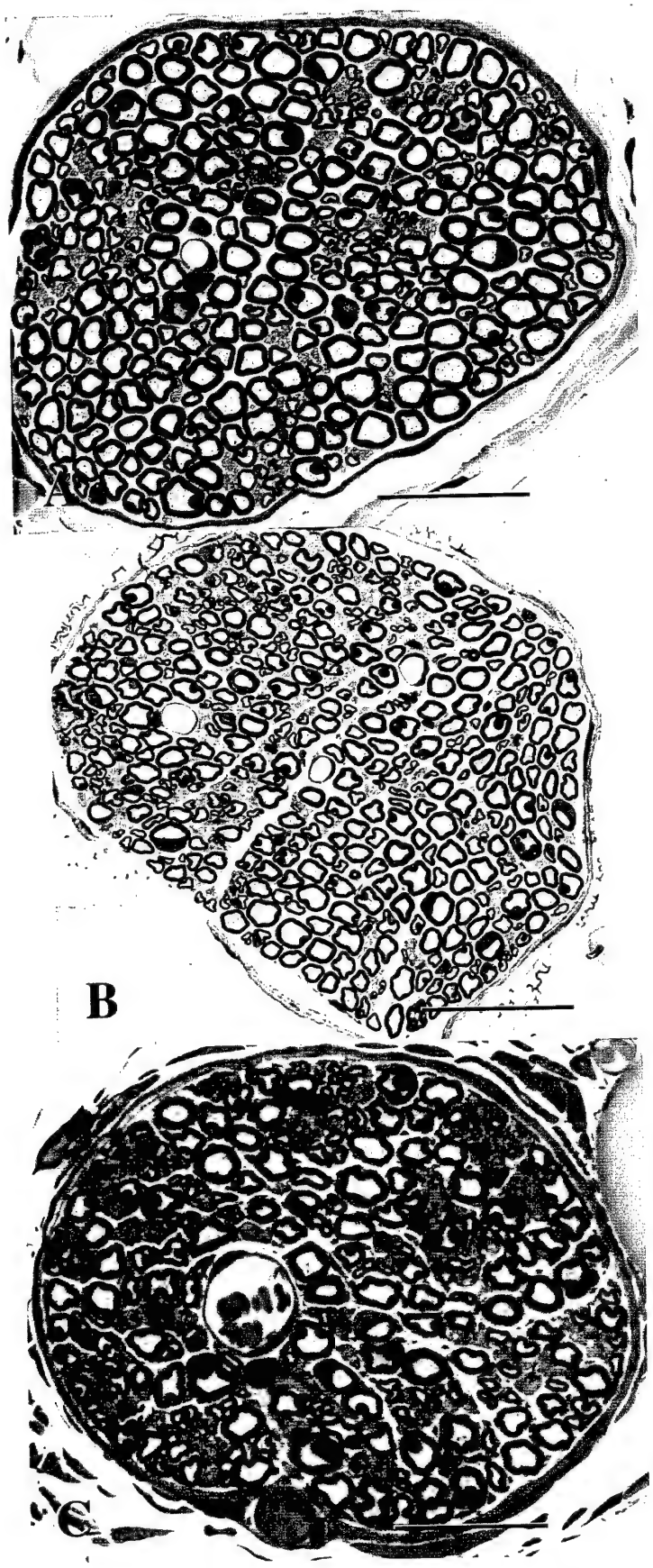
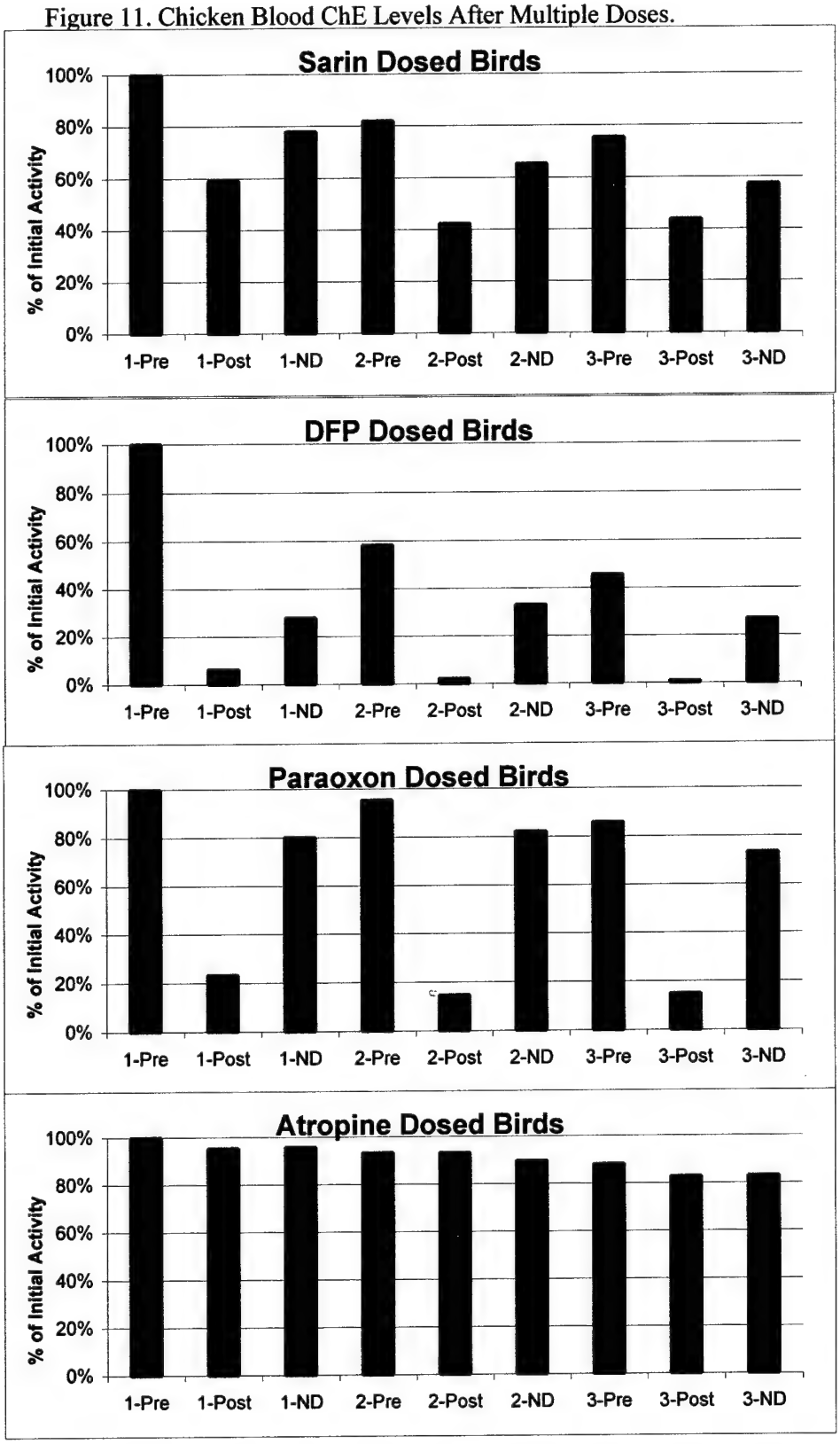


Figure 10. Cross sections of mouse distal sural nerves from the same level (ankle) of mice treated with TOCP. All of these nerves appear normal, with only preparation artifacts evident. **A**. Control mouse distal sural nerve (Bar = 38 μm). **B**. Distal sural nerve dosed with 3.2 g/kg TOCP (Bar = 47 μm). **C**. Distal sural nerve dosed with 6.4 g/kg TOCP (Bar = 38 μm).

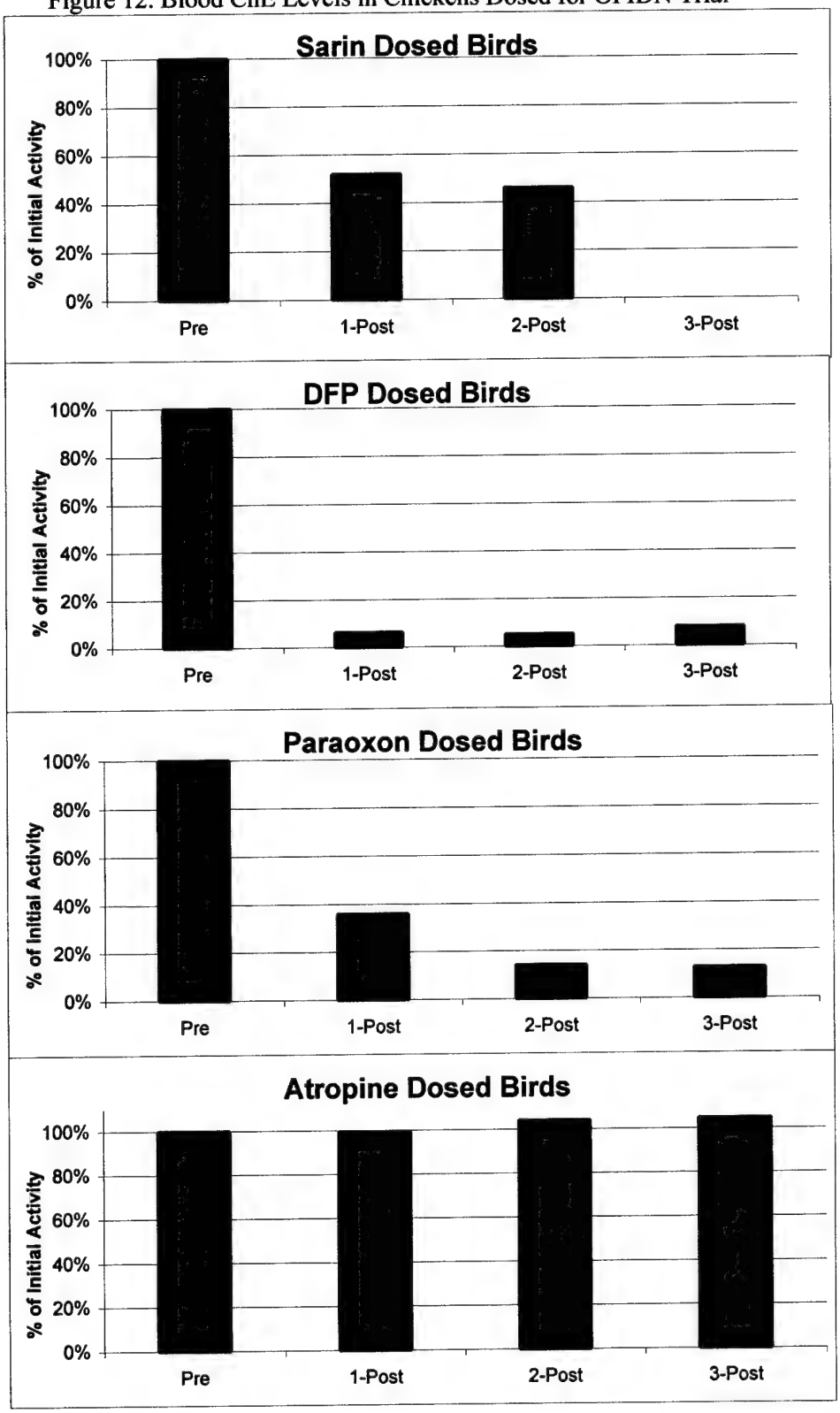
Figure 11. Chicken Blood ChE Levels After Multiple Doses.



Values are average from paired birds.

Pre = Pre-Dose, Post = ~1 Hr Post-Dose, ND = Next Day (~24 Hr) of Dose 1, 2, 3.

Figure 12. Blood ChE Levels in Chickens Dosed for OPIDN Trial



Pre = Blood taken before the first dose; Post = ~1 Hr after doses 1, 2, 3.

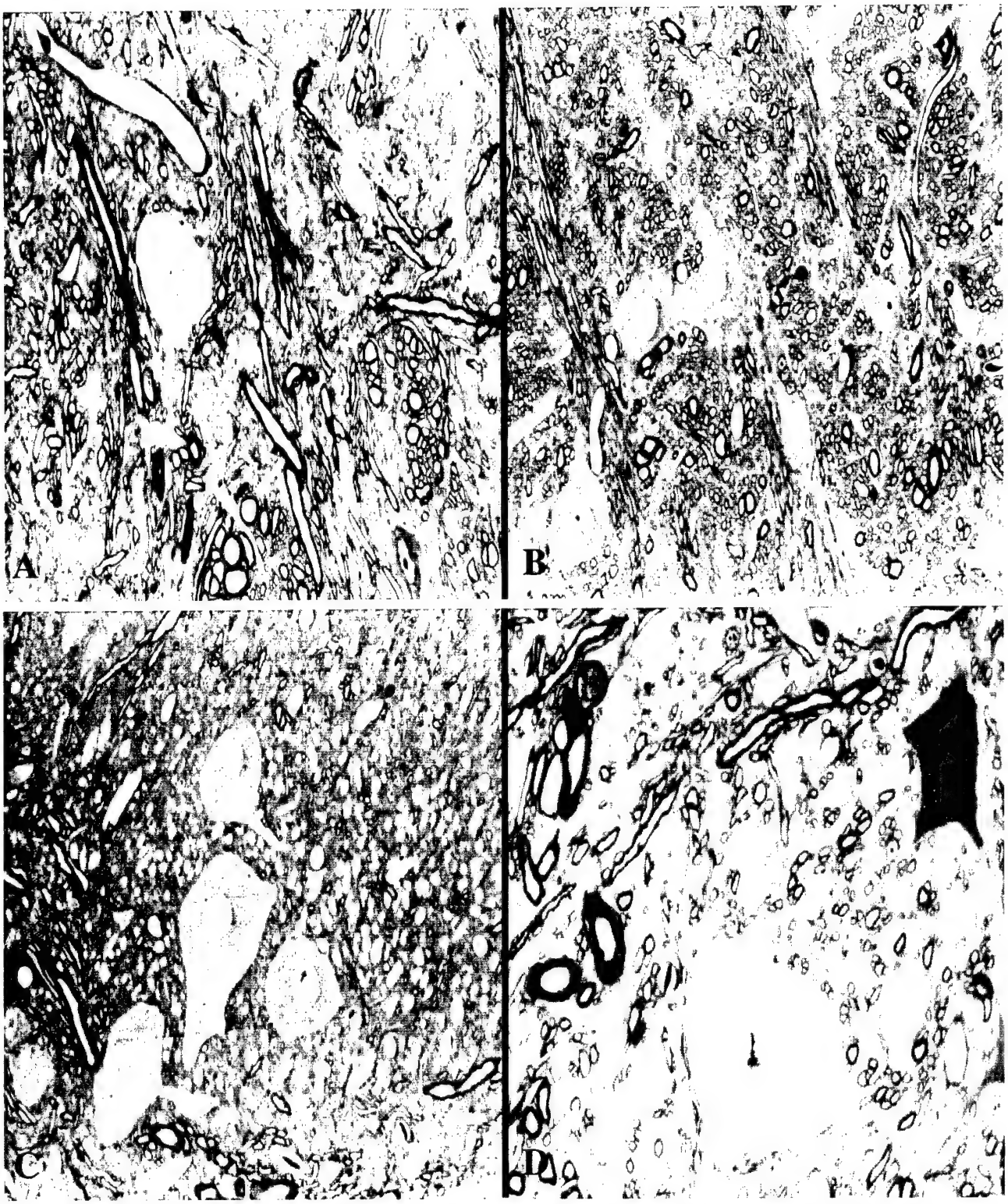


Figure 13. **A**. Medulla oblongata from animal #14702 showing several large neurons. **B**. Spinal cord lumbar region from animal #14711. Note the large normal neuron (lower center), and one dense necrotic neuron (upper right). **C**. Medulla oblongata from animal # 14716 showing large neuron. **D**. Medulla oblongata from animal # 14707.

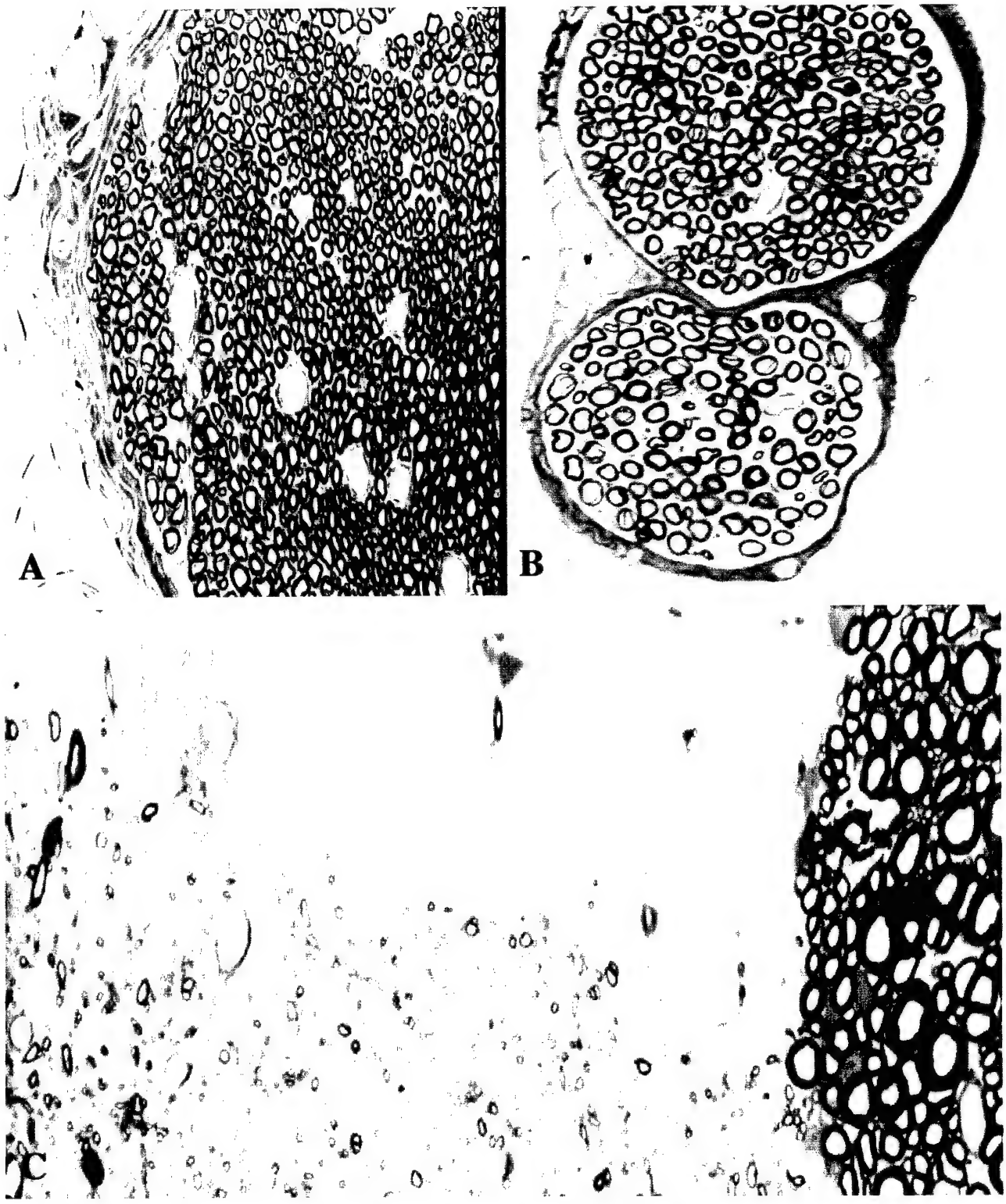


Figure 14. **A.** Tibial nerve distal from animal # 14711. **B.** Sural nerve distal from animal #14734. **C.** Spinal cord from the lumbar region of animal # 14711. Note adipose tissue (upper center), next to a spinal nerve track.

Figure 15. Effects of Multiple Dosing on Chicken Weight

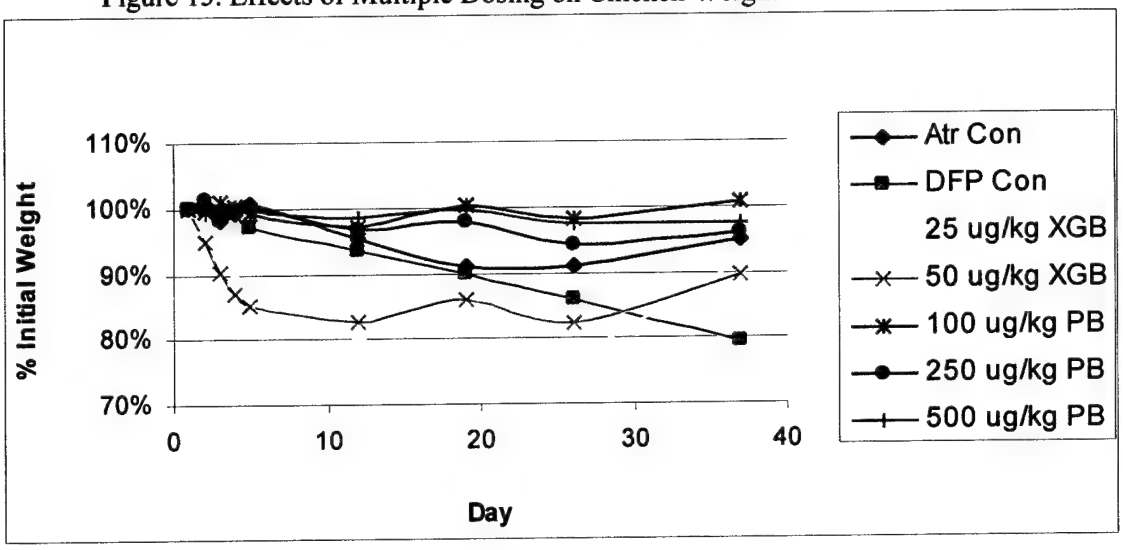


Figure 16. Inhibition and Recovery of Plasma ChE

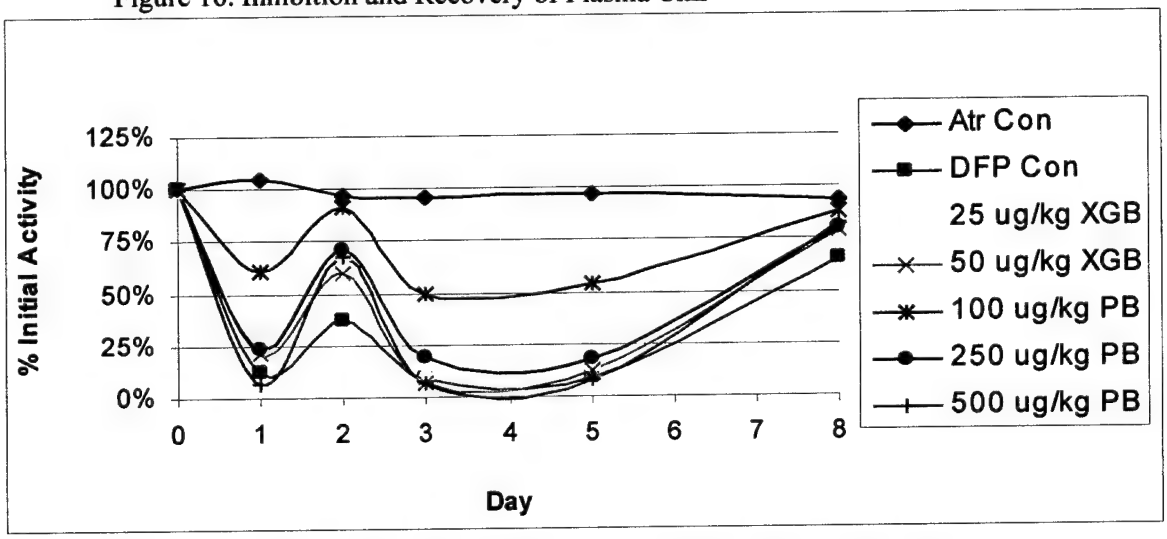
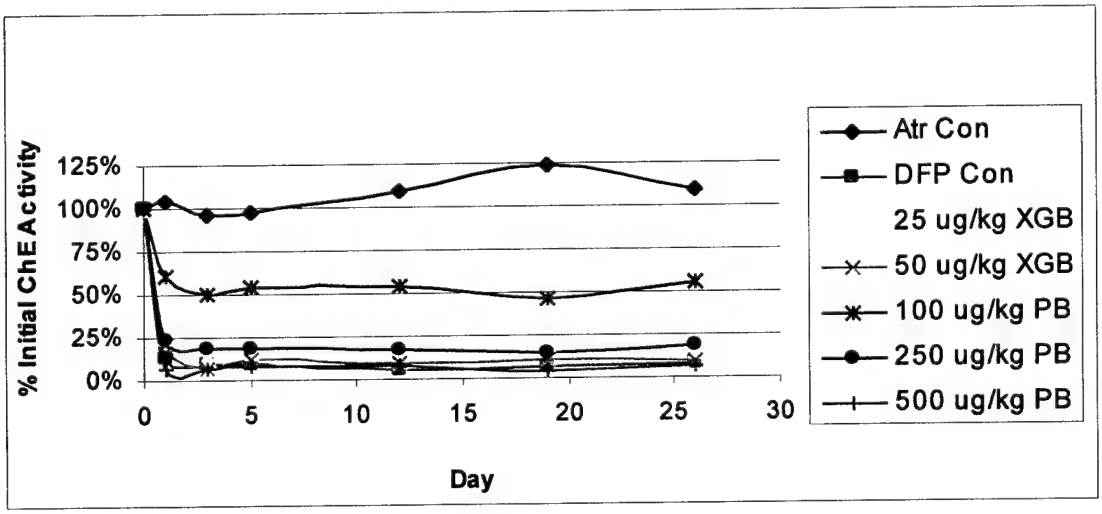


Figure 17. Effects of Multiple Exposures on Chicken Plasma ChE Activity



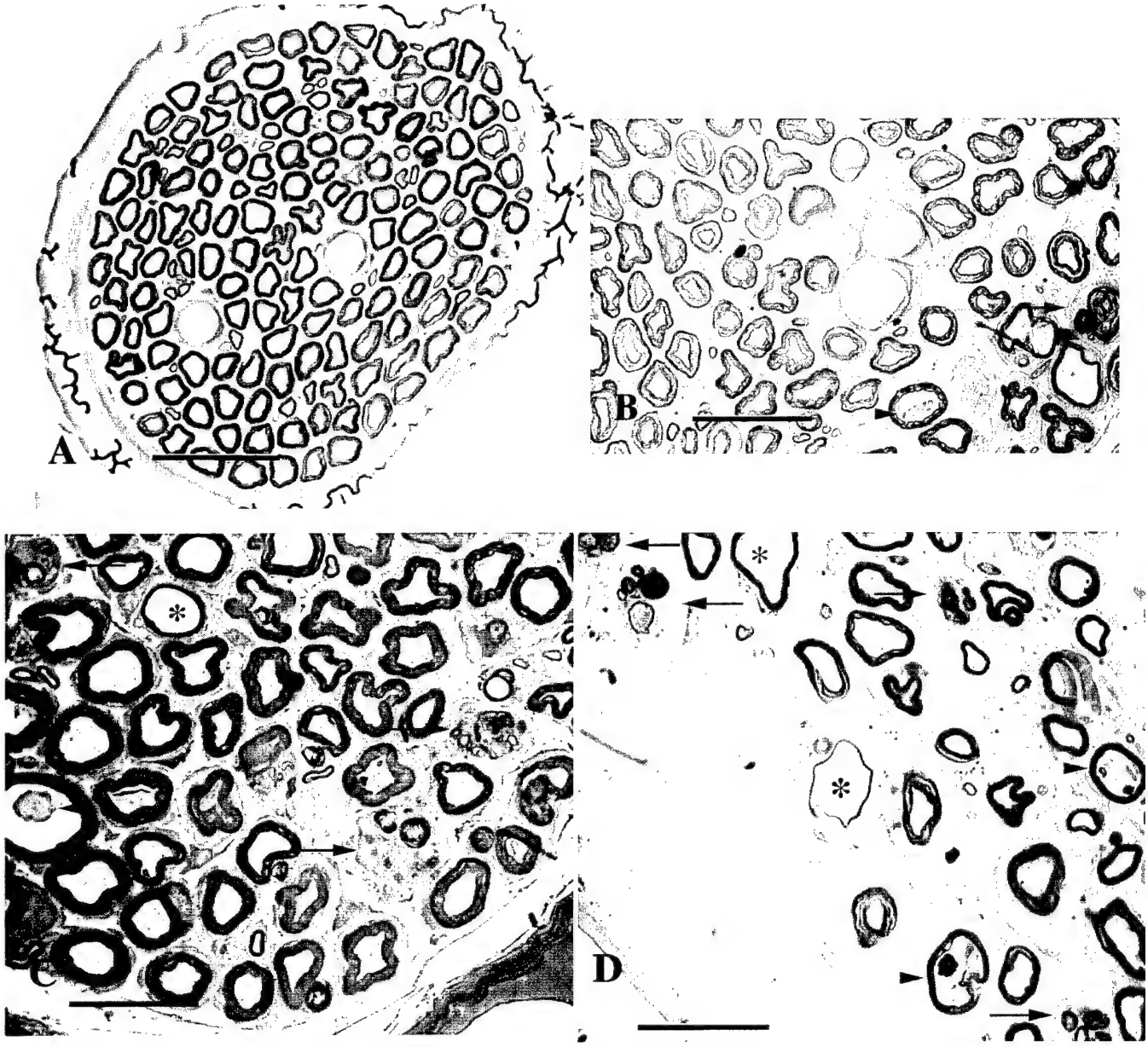
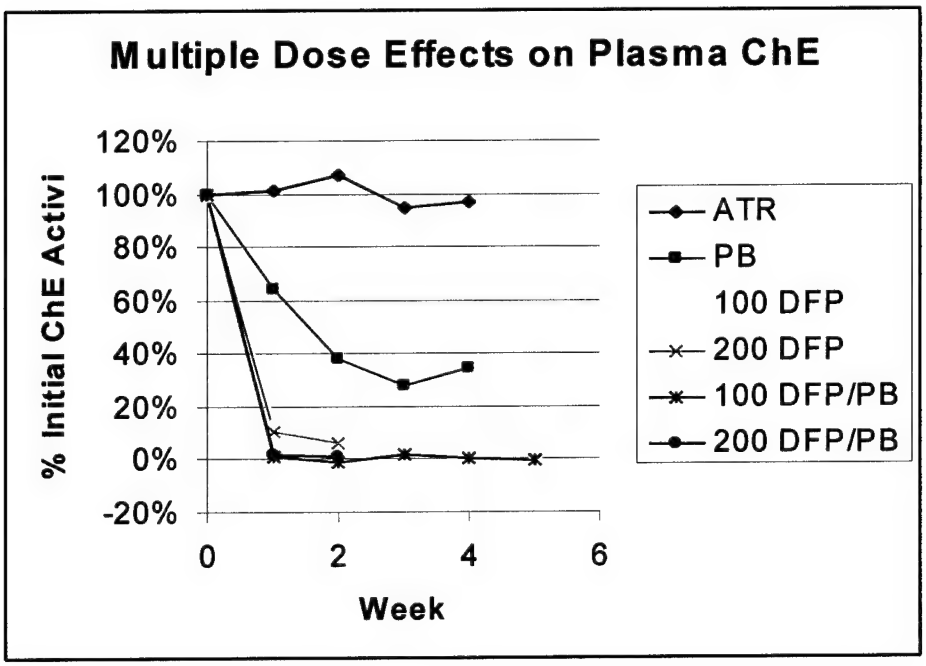


Figure 18. Distal sural nerves in cross section from hens taken from the same level (ankle) demonstrating increasing levels of neuropathy. **A**. Distal sural nerve from hen # 14221, showing normal morphology (stage 0-1) (bar = 48 μm). **B**. Distal sural from hen #14767 showing remains of single large-diameter myelinated nerve fiber delimited by basal lamina (arrow), also seen is evidence of primary axonal degeneration (arrowhead) characterized by large number of vesicular structures within the axon. Note that large numbers of myelinated nerve fibers are normal in appearance (stage 2) (bar = 26 μm). **C**. Distal sural from hen (no band) showing both stages of degeneration as B & C (arrows, and arrowheads) and also a myelinated axon swelling with subsequent thinning of the myelin sheath (asterisk). Note the increasing number of degenerating nerves (stage 3) (bar = 24 μm). **D**. Distal sural nerve from hen # 4232 demonstrating increasing numbers of degenerating nerves of all three types of degeneration mentioned in A, B ,& C (stage 4 pathology) (bar = 42 μm).

Figure 19. ChE Inhibition in Hens Treated with DFP +/- PB



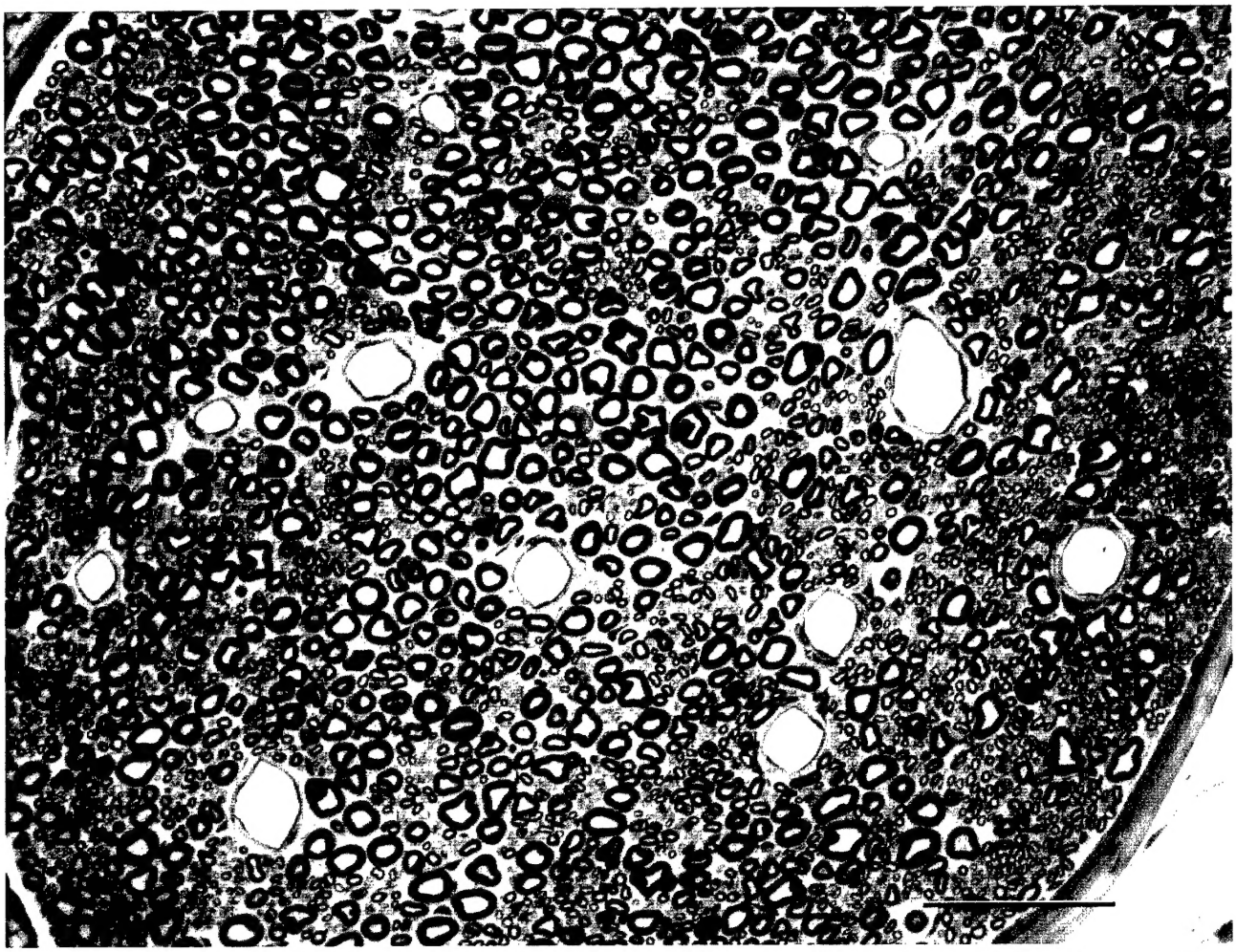


Figure 20. Cross section of the distal peroneal nerve of hen treated with 100 μg/kg PB. No pathology evident (Bar = 59 μm).

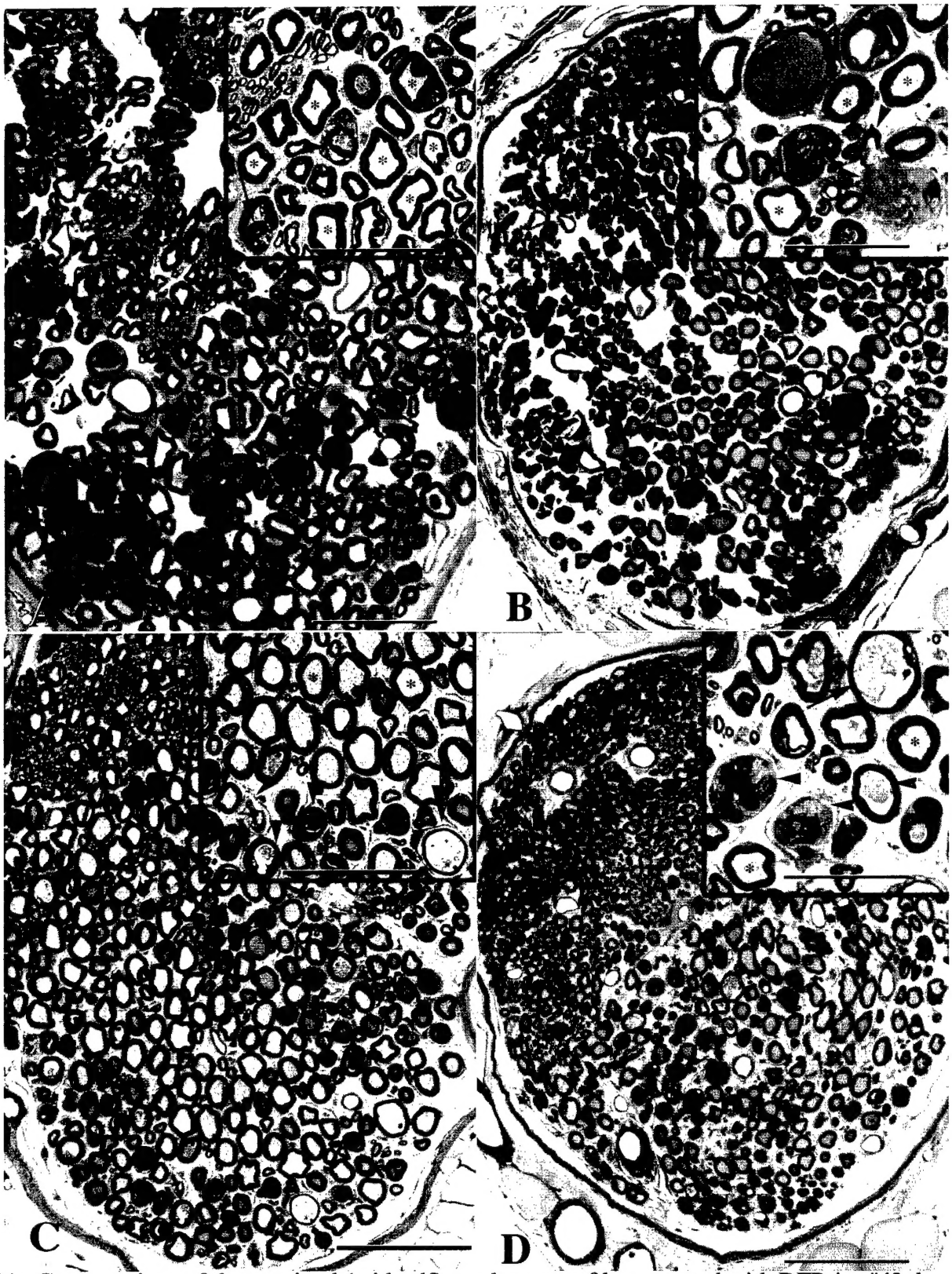


Figure 21. Cross sections of the proximal (mid calf) sural nerves of hens treated with DFP **A.** #43 dosed at 100mg/kg DFP showing stage 2 pathology (Bar = 62 μm). Inset shows degenerating axons (arrows). Asterisks show preserved fibers (stage 2) (Bar = 40 μm). **B.** #34 dosed at 100mg/kg DFP & 100μg/kg PB (Bar = 60 μm). Inset shows degenerating myelinated nerve fibers (arrows). Note the myelinated nerve fibers that appear normal (asterisks) (Bar = 42 μm). **C.** #32 dosed at 200mg/kg DFP showing stage 3 pathology (Bar = 75 μm). Inset shows degenerating myelinated nerve fibers (arrows) Note the number of myelinated nerve fibers that appear normal (asterisks) (Bar = 42 μm). **D.** #36 dosed with 200mg/kg DFP & 100μg/kg PB showing stage 2 axonal degeneration (Bar = 87 μm). Inset shows degenerating myelinated nerve fibers (arrows). Note the number of myelinated nerve fibers that appear normal (asterisks) (Bar = 29 μm).

Figure 22. Effects of Multiple Exposures on Chicken Plasma - Histology Trial

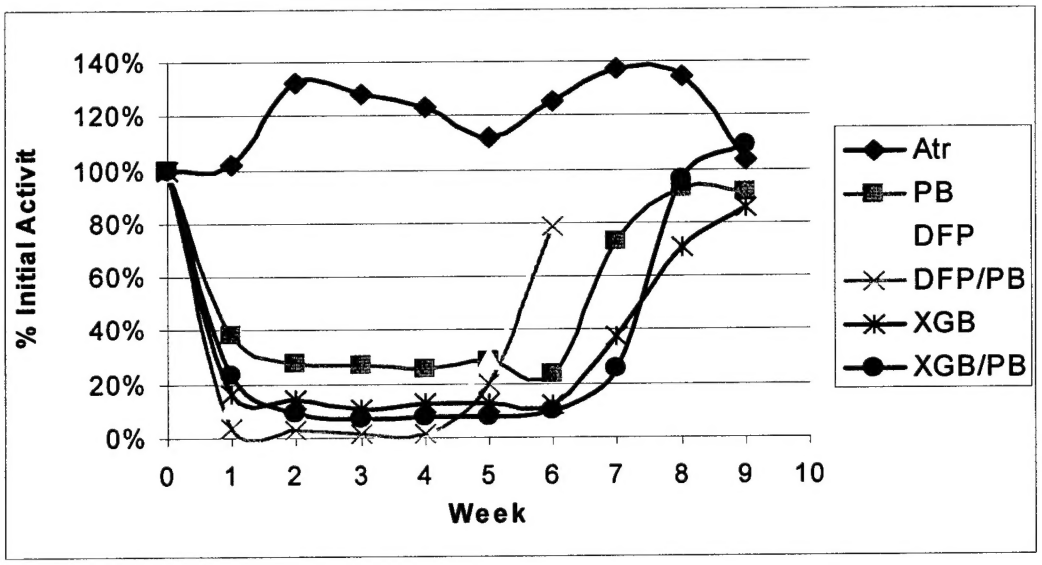


Figure 23. Plasma ChE Activity After First Treatment - Histology Trial

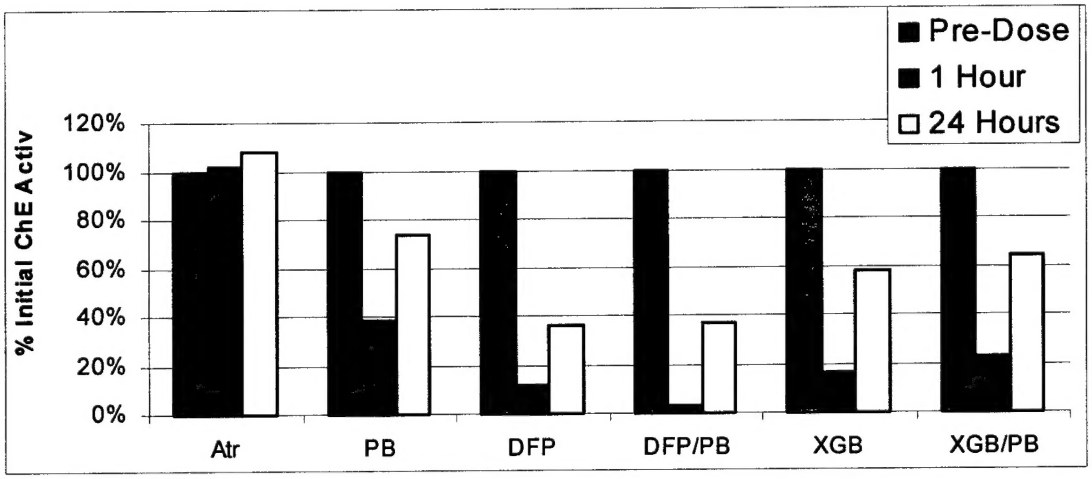


Figure 25. Effects of Multiple Doses on Brain AChE Activity

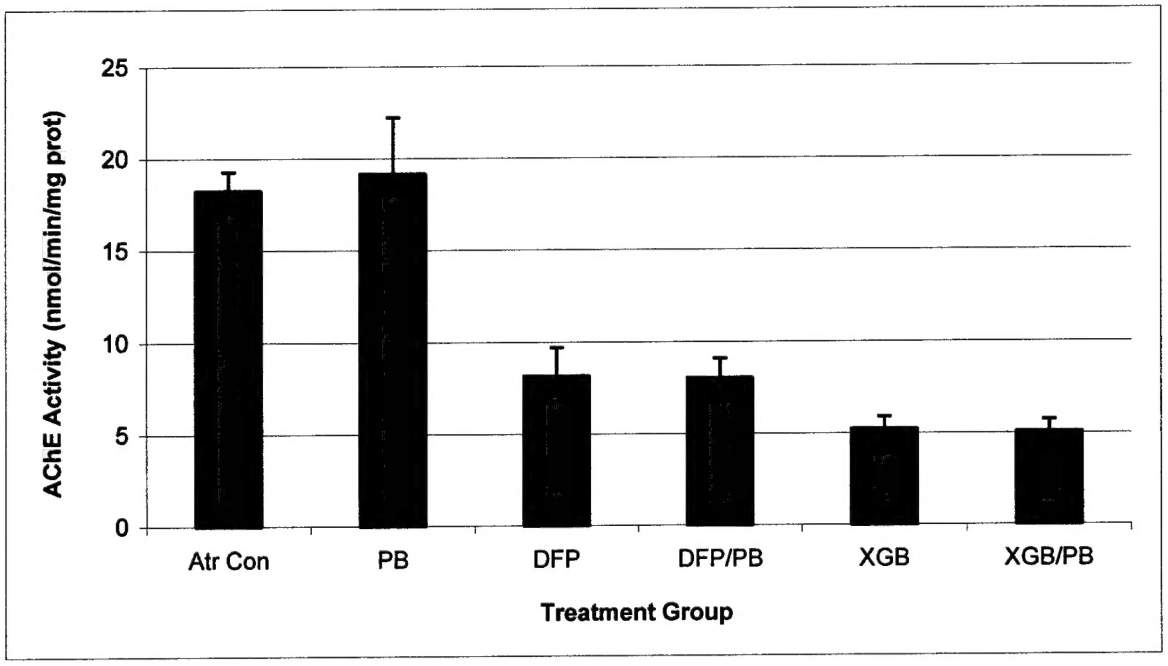


Figure 26. Effects of Multiple Doses on Brain NTE Activity